8434143

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

November 21, 2023

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APPLICATION NUMBER: *12/091,896*
FILING DATE: *November 03, 2008*
PATENT NUMBER: *8283522*
ISSUE DATE: *October 09, 2012*

Certified by

Performing the Functions and Duties of the
Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 12091896 Document Date: 04/28/2008

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- Sequence Listing

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Form Revision Date: February 8, 2006

Corteva Exhibit 2014 - Page 2 of 1122
Inari v. Corteva - PGR2023-00022

April 28, 2008

PRELIMINARY AMENDMENT
Patent Application
Docket No. DAS-129XC1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry R. Wright, Justin M. Lira, Terence Anthony Walsh, Donald J. Merlo, Pon Samuel Jayakumar, Gaofeng Lin
Docket No.	:	DAS-129XC1
For	:	Novel Herbicide Resistance Genes

Mail Stop PCT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Sir:

It is respectfully requested that the above-identified patent application be amended as follows:

J:\DAS\DAS-129XC1\PTO\PreAmd.doc/DNB/mrc

In the Specification

Please insert the following new paragraph after the Title of the invention on page 1, line 1:

Cross-Reference to Related Application

This application is the U.S. national stage application of International Patent Application No. PCT/US2006/042133, filed October 27, 2006, which claims the benefit of U.S. Provisional Patent Application No. 60/731,044, filed October 28, 2005, the disclosures of which are hereby incorporated by reference in their entireties, including all figures, tables and amino acid or nucleic acid sequences.

After page 153: Please insert as new page 154 the attached Abstract of the Disclosure.

J:\DAS\DAS-129XC1\PTO\PreAmd.doc/DNB/mrc

In the Claims

Please cancel claims 1-88. Please add the following claims:

89. A plant cell comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said stringent conditions comprise 0.2x SSPE at 65° C.

90. A plant comprising a plurality of cells of claim 89.

91. A method of controlling weeds in a crop field, said method comprising applying an arlyoxyalkanoate herbicide to said crop field, said crop field comprising a plurality of plants, each said plant comprising a plurality of plant cells of claim 89, wherein expression of said polynucleotide renders said plant resistant or tolerant to said arlyoxyalkanoate herbicide.

92. The method of claim 91 wherein said arlyoxyalkanoate herbicide is 2,4-D.

93. The method of claim 91 wherein said plants are dicots.

94. The method of claim 93 wherein said dicots are soybean plants.

95. The method of claim 91 wherein said method further comprises applying said arlyoxyalkanoate herbicide to said crop field prior to planting seeds in said field.

96. The method of claim 91 wherein said method further comprises applying said arlyoxyalkanoate herbicide to said crop field after seeds are planted in said field but prior to emergence of said plants grown from said seeds.

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97. The method of claim 91 wherein said method further comprises applying glyphosate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glyphosate.

98. The method of claim 91 wherein said method further comprises applying glufosinate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glufosinate.

99. The method of claim 91 wherein said method further comprises applying glyphosate and glufosinate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glyphosate, and wherein said plants further comprise a third polynucleotide wherein expression of said third polynucleotide renders said plants resistant or tolerant to glufosinate.

100. The plant cell of claim 89 wherein said protein has at least 85% amino acid sequence identity with SEQ ID NO:2 or SEQ ID NO:4.

101. A seed comprising a plant cell of claim 89.

102. The plant of claim 90 wherein said plant further comprises an insect-resistance gene.

103. A polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said polynucleotide comprises a codon composition that is optimized for expression in a plant, and wherein said stringent conditions comprise 0.2x SSPE at 65° C.

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Inari v. Corteva - PGR2023-00022

Remarks

In order to reduce filing fees, the subject claims are being pursued at this time. The applicants expressly reserve the right to pursue any additional (non-exclusive) claims filed with the corresponding PCT application.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16, 1.17, and 1.492 as required by this paper to Deposit Account No. 19-0065.

Respectfully submitted,

Jay M. Sanders
Patent Attorney
Registration No. 39,355
Phone No.: 352-375-8100
Fax No.: 352-372-5800
Address: P.O. Box 142950
Gainesville, FL 32614-2950

JMS/mrc
Attachment: Abstract of the Disclosure

J:\DAS\DAS-129XC1\PTO\PreAmd.doc/DNB/mrc

Abstract of the Disclosure

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide tolerant crop trait and selectable marker opportunities.

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Inari v. Corteva - PGR2023-00022

Application Information

Application Type::	Regular (National Stage)
Subject Matter::	Utility
Suggested Classification::	None
Suggested Group Art Unit::	None
CD-ROM or CD-R?::	None
Number of CD disks::	None
Number of copies of CDs::	None
Sequence submission?::	Paper
Computer Readable Form?::	Yes
Number of Copies of CRF::	None
Title::	NOVEL HERBICIDE RESISTANCE GENES
Attorney Docket Number::	DAS-129XC1
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	None
Total Drawing Sheets::	3
Small Entity?::	No
Petition included?::	No
Petition Type::	N/A
Secrecy Order in Parent Appl.?::	No

Corteva Exhibit 2014 - Page 9 of 1122
Inari v. Corteva - PGR2023-00022

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Unknown
Inventor One Given Name::	Terry R.
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City of Mailing Address::	Westfield
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46074

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Unknown
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Country of Residence::	US
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City of Mailing Address::	Fishers
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46038

Corteva Exhibit 2014 - Page 10 of 1122
Inari v. Corteva - PGR2023-00022

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
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Inventor Three Given Name::	Terence Anthony
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Country of Residence::	US
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City of Mailing Address::	Zionsville
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46077

Applicant Information

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State or Province of Residence::	IN
Country of Residence::	US
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City of Mailing Address::	Carmel
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46032

Corteva Exhibit 2014 - Page 11 of 1122
Inari v. Corteva - PGR2023-00022

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
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Inventor Five Given Name::	Pon Samuel
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Country of Residence::	US
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City of Mailing Address::	Carmel
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46032

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Unknown
Inventor Six Given Name::	Gaofeng
Family Name::	LIN
City of Residence::	Zionsville
State or Province of Residence::	IN
Country of Residence::	US
Street of Mailing Address::	981 Franklin Trace
City of Mailing Address::	Zionsville
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46077

Corteva Exhibit 2014 - Page 12 of 1122
Inari v. Corteva - PGR2023-00022

Representative Information

Representative Customer Number::	000023557

Correspondence Information

Correspondence Customer Number::	000023557
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Telephone Number Two::	
Fax Number::	(352) 372-5800
Electronic Mail Address::	jms@slspatents.com

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This application is a	National Stage of	PCT/US2006/042133	October 27, 2006
PCT/US2006/042133	An application claiming the benefit under 35 USC 119(e) of	60/731,044	October 28, 2005

Assignee Information

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City of Mailing Address::	Indianapolis
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46268-1054

Corteva Exhibit 2014 - Page 13 of 1122
Inari v. Corteva - PGR2023-00022

April 28, 2008

Patent Application
Docket No. DAS-129XC1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry R. Wright, Justin M. Lira, Terence Anthony Walsh, Donald J. Merlo, Pon Samuel Jayakumar, Gaofeng Lin
Docket No.	:	DAS-129XC1
For	:	Novel Herbicide Resistance Genes

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBMISSION OF SEQUENCE LISTING
AND STATEMENT UNDER 37 C.F.R. §§1.821-1.825

Sir:

Transmitted herewith is an application containing a sequence listing under 37 C.F.R. §§1.821-1.825.

The sequence is submitted in computer readable format and on paper. I hereby certify that the paper and computer readable copies contain the same information.

Respectfully submitted,

Jay M. Sanders
Patent Attorney
Registration No. 39,355
Telephone No.: (352) 375-8100
Facsimile No.: (352) 372-5800
Address: Saliwanchik, Lloyd & Saliwanchik
A Professional Association
P.O. Box 142950
Gainesville, FL 32614-2950

JMS/jb/mrc
Attachments: Sequence listing on paper and computer readable format (.txt format)

J:\DAS\DAS-129XC1\PTO\SubSeqList.doc/DNB/jb/mrc

SEQUENCE LISTING

<110> Wright, Terry R.
Lira, Justin M.
Walsh, Terence A.
Merlo, Donald J.
Jayakumar, Pon Samuel
Lin, Gaofeng

<120> Novel Herbicide Resistance Genes

<130> DAS-129XC1

<150> US 60/731,044
<151> 2005-10-28

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Leu Leu Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala
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Glu Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala
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Electronic Patent Application Fee Transmittal	
Application Number:	
Filing Date:	
Title of Invention:	Novel Herbicide Resistance Genes
First Named Inventor/Applicant Name:	Terry R. Wright
Filer:	Jay M. Sanders/Mary Calderwood
Attorney Docket Number:	DAS-129XC1
Filed as Large Entity	

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
National Stage Fee	1631	1	310	310
Natl Stage Search Fee - U.S. was the ISA	1641	1	100	100
National Stage Exam - all other cases	1633	1	210	210
Pages:				
Natl Stage Appl Sz fee per 50 pgs >100	1681	2	260	520
Claims:				
Miscellaneous-Filing:				
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1140**

Electronic Acknowledgement Receipt	
EFS ID:	3220792
Application Number:	12091896
International Application Number:	PCT/US06/42133
Confirmation Number:	6428
Title of Invention:	Novel Herbicide Resistance Genes
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	23557
Filer:	Jay M. Sanders/Mary Calderwood
Filer Authorized By:	Jay M. Sanders
Attorney Docket Number:	DAS-129XC1
Receipt Date:	28-APR-2008
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(54) Title: NOVEL HERBICIDE RESISTANCE GENES

(57) Abstract: The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide tolerant crop trait and selectable marker opportunities.

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NOVEL HERBICIDE RESISTANCE GENES

Background of the Invention

[0001] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0002] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant soybeans, for example, can be sprayed to control weeds without significantly damaging the soybean plants.

[0003] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. Alfalfa was the first perennial GTC introduced, furthering the opportunity for repeated use of glyphosate on the same crop and fields repeatedly over a period of years. More GTCs (*e.g.,* wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (*e.g.*, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; *see* "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0004] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection

pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

[0005] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Powles and Preston, 2006, Ng *et al.*, 2003; Simarmata *et al.*, 2003; Lorraine-Colwill *et al.*, 2003; Sfiligoj, 2004; Miller *et al.*, 2003; Heap, 2005; Murphy *et al.*, 2002; Martin *et al.*, 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past five years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Sorghum halepense, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis, Plantago lanceolata, Amaranthus palmerii, and Amaranthus rudis.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0006] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting

no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2005).

[0007] 2,4-D is in the phenoxy acid class of herbicides, as is MCPA. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known. Triclopyr and fluroxypyr are pyridyloxyacetic acid herbicides whose mode of action is as a synthetic auxin, also.

[0008] These herbicides have different levels of selectivity on certain plants (*e.g.,* dicots are more sensitive than grasses). Differential metabolism by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

[0009] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D currently formulated has a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0010] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0011] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many

homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0012] One unique example with low homology to *tfdA* (31% amino acid identity) is *sdpA* from *Delftia acidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002, Westendorf *et al., 2003*). This enzyme has been shown to catalyze the first step in (S)-dichlorprop (and other (S)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Transformation of this gene into plants, has not heretofore been reported.

[0013] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0014] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxyacetate auxins (such as 2,4-D and dichlorprop), pyridyloxyacetate auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. A multifunctional enzyme for the degradation of herbicides covering multiple modes of action has recently been described (PCT US/2005/014737; filed May 2, 2005). Another unique multifunctional enzyme and potential uses are described hereafter.

Brief Summary of the Invention

[0015] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of

the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, ALS- (imidazolinone, sulfonylurea), aryloxyalkanoate-, HPPD-, PPO-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0016] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, MCPA, triclopyr, fluroxypyr, and one or more commercially available herbicides (*e.g.*, glyphosate, glufosinate, paraquat, ALS-inhibitors (e.g., sulfonylureas, imidazolinones, triazolopyrimidine sulfonanilides, et al), HPPD inhibitors (e.g, mesotrione, isoxaflutole, *et al.*), dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0017] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide-tolerant crop (HTC) trait and selectable marker opportunities. Plants of the subject invention can be resistant throughout their entire life cycle.

[0018] There was no prior motivation to produce plants comprising an *AAD-12* gene (preferably an *AAD-12* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-12 enzyme to render the plants resistant a phenoxyacetic acid herbicide (such as 2,4-D) and/or one or more pyridyloxyacetates herbicides such as triclopyr and fluroxypyr. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0019] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxyacetate auxin and/or pyridyloxyacetates auxin herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid and other aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more pyridyloxyacetate or phenoxyacetate auxin herbicides to plants comprising an *AAD-12* gene. The subject invention also provides methods of using an *AAD-12* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-12*, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0020] **Figure 1** illustrates the general chemical reaction that is catalyzed by AAD-12 enzymes of the subject invention.

[0021] **Figure 2** is an amino acid sequence alignment of an exemplified AAD-12 protein, TfdA, AAD-2, AAD-1, and TauD.

[0022] **Figure 3** illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop.

Brief Description of the Sequences

[0023] **SEQ ID NO:1** is the nucleotide sequence of *AAD-12* from *Delftia acidovorans*.

[0024] **SEQ ID NO:2** is the translated protein sequence encoded by SEQ ID NO:1.

[0025] **SEQ ID NO:3** is the plant optimized nucleotide sequence of *AAD-12 (v1)*.

[0026] **SEQ ID NO:4** is the translated protein sequence encoded by SEQ ID NO:3.

[0027] **SEQ ID NO:5** is the E.coli optimized nucleotide sequence of *AAD-12 (v2)*.

[0028] **SEQ ID NO:6** is the sequence of the M13 forward primer.

[0029] **SEQ ID NO:7** is the sequence of the M13 reverse primer.

[0030] **SEQ ID NO:8** is the sequence of the forward *AAD-12 (v1)* PTU primer.

[0031] **SEQ ID NO:9** is the sequence of the reverse *AAD-12 (v1)* PTU primer.

[0032] **SEQ ID NO:10** is the sequence of the forward *AAD-12 (v1)* coding PCR primer.

[0033] **SEQ ID NO:11** is the sequence of the reverse *AAD-12 (v1)* coding PCR primer.

[0034] **SEQ ID NO:12** shows the sequence of the "sdpacodF" *AAD-12 (v1)* primer.

[0035] **SEQ ID NO:13** shows the sequence of the "sdpacodR" *AAD-12 (v1)* primer.

[0036] **SEQ ID NO:14** shows the sequence of the "Nco1 of Brady" primer.

[0037] **SEQ ID NO:15** shows the sequence of the "Sac1 of Brady" primer.

Detailed Description of the Invention

[0038] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of the subject herbicide tolerance trait for 2,4-D is its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the *AAD-12* gene is that unlike all *tfdA* homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (*e.g.*, triclopyr, fluroxypyr) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). *See* **Table 1**. A general illustration of the chemical reactions catalyzed by the subject AAD-12 enzyme is shown in **Figure 1**. (Addition of O_2 is stereospecific; breakdown of intermediate to phenol and glyoxylate is spontaneous.) It should be understood that the chemical structures in Figure 1 illustrate the molecular backbones and that various R groups and the like (such as those

shown in Table 1) are included but are not necessarily specifically illustrated in Figure 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the *AAD-12* gene in plants affords protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled. The subject invention can also be used to protect from drift or other off-site synthetic auxin herbicide injury for the full breadth of commercially available phenoxy auxins. **Table 1** defines commercially available pyridyloxy and phenoxy auxins and provides relevant chemical structures.

Table 1. Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

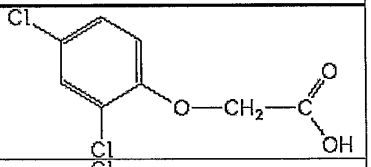
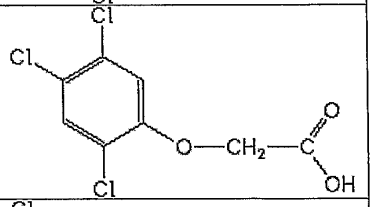
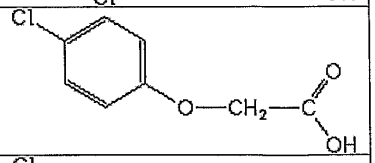
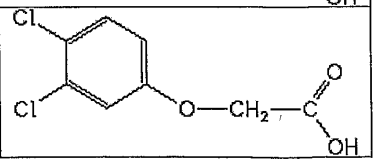
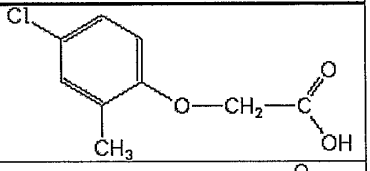
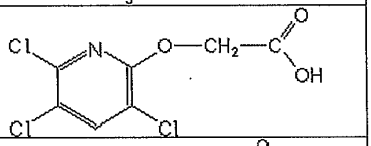
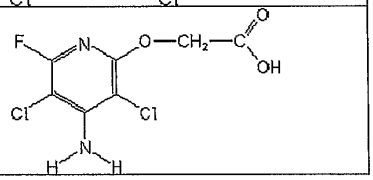
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	

Table 1. Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
MCPA	94-74-6	25 – 4000	125 - 1550	
Triclopyr	55335-06-3	50 – 2000	70 – 840	
Fluroxypyr	69377-81-7	25 – 2000	35 – 560	

[0039] A single gene (*AAD-12*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-12* can provide protection *in planta* to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing *AAD-12* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

[0040] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-12* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (*e.g.*, 2,4-D) and pyridyloxyacetates auxin herbicides (*e.g.*, triclopyr)—selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0041] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/*AAD-12* stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0042] Plantlets are typically resistant throughout the entire growing cycle. Transformed plants will typically be resistant to new herbicide application at any time the gene is expressed. Tolerance is shown herein to 2,4-D across the life cycle using the constitutive promoters tested thus far (primarily CsVMV and AtUbi 10). One would typically expect this, but it is an improvement upon other non-metabolic activities where tolerance can be significantly impacted by the reduced expression of a site of action mechanism of resistance,

for example. One example is Roundup Ready cotton, where the plants were tolerant if sprayed early, but if sprayed too late the glyphosate concentrated in the meristems (because it is not metabolized and is translocated); viral promoters Monsanto used are not well expressed in the flowers. The subject invention provides an improvement in these regards.

[0043] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants, surfactants, drift retardants, or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0044] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS,* glyphosate oxidoreductase (*GOX*), *GAT*), glufosinate resistance (*e.g.*, *Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g., PPO-1*), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), dicamba-degrading enzymes (*see, e.g.,* US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes. *In vivo* modified EPSPS can be used in some preferred embodiments, as well as Class I, Class II, and Class III glyphosate resistance genes.

[0045] Regarding additional herbicides, some additional preferred ALS inhibitors include but are not limited to the sulfonylureas (such as chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron), imidazoloninones (such as imazamox, imazethapyr, imazaquin), triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam),

pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include but are not limited to mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include but are not limited to flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0046] Additionally, *AAD-12* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0047] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-1 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

[0048] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0049] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and

pyridyloxyacetate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic acids, and pyridyloxyacetic acid herbicides by a recombinantly expressed AAD-12 enzyme.

[0050] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP *in vitro*. An additional advantage that *AAD-12* transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0051] The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an *AAD-12* gene.

[0052] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0053] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0054] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-

ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0055] The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, *sdpA*, from *Delftia acidivorans* (Westendorf *et al.*, 2002, 2003) with low homology to *tfdA* (31% amino acid identity). This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. *SdpA* has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0056] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

[0057] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and/or pyridyloxy auxin herbicides.

[0058] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. *AAD-12* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits [*e.g.*, glyphosate resistance, glufosinate resistance, ALS-inhibitor (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide) resistance, bromoxynil resistance, HPPD-

inhibitor resistance, PPO-inhibitor resistance, *et al.*], and insect resistance traits (*Cry1F, Cry1Ab, Cry 34/45*, other Bt. Proteins, or insecticidal proteins of a non-*Bacillis* origin, *et al.*) for example. Additionally, *AAD-12* can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0059] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-12*-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention.

[0060] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[0061] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

[0062] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[0063] The transgenic expression of the subject *AAD-12* genes is exemplified in, for example, *Arabidopsis*, tobacco, soybean, cotton, rice, corn and canola. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as pasture grasses or turf grass) and dicot

crops like alfalfa, clover, tree species, *et al.* Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

[0064] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

[0065] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

[0066] Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention

are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[0067] One cannot easily discuss the term "resistance" and not use the verb "tolerate" or the adjective "tolerant." The industry has spent innumerable hours debating Herbicide Tolerant Crops (HTC) versus Herbicide Resistant Crops (HRC). HTC is a preferred term in the industry. However, the official Weed Science Society of America definition of resistance is "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." As used herein unless otherwise indicated, herbicide "resistance" is heritable and allows a plant to grow and reproduce in the presence of a typical herbicidally effective treatment by a herbicide for a given plant, as suggested by the current edition of *The Herbicide Handbook* as of the filing of the subject disclosure. As is recognized by those skilled in the art, a plant may still be considered "resistant" even though some degree of plant injury from herbicidal exposure is apparent. As used herein, the term "tolerance" is broader than the term "resistance," and includes "resistance" as defined herein, as well an improved capacity of a particular plant to withstand the various degrees of herbicidally induced injury that typically result in wild-type plants of the same genotype at the same herbicidal dose.

[0068] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

[0069] The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[0070] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[0071] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria.

[0072] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutant strains can also be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[0073] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[0074] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook *et al.*, 1989, which is incorporated herein by reference.

[0075] Polynucleotides and probes. The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[0076] The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-12* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage. In addition, desired levels and times of expression can also depend on the type of plant and the level of tolerance desired. Some preferred embodiments use strong constitutive promoters combined with transcription enhancers and the like to increase expression levels and to enhance tolerance to desired levels. Some such applications are discussed in more detail below, before the Examples section.

[0077] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or

"coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[0078] In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

[0079] Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

[0080] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

[0081] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[0082] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[0083] Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

[0084] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

Tm = 81.5° C + 16.6 Log[Na+] + 0.41(%G+C) - 0.61(%formamide) - 600/length of duplex in base pairs.

[0085] Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

[0086] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

Tm (°C) = 2(number T/A base pairs) + 4(number G/C base pairs)

(Suggs *et al.*, 1981).

[0087] Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

[0088] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low:	1 or 2x SSPE, room temperature
Low:	1 or 2x SSPE, 42° C
Moderate:	0.2x or 1x SSPE, 65° C
High:	0.1x SSPE, 65° C.

[0089] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[0090] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.,* 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[0091] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the

scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[0092] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein.

[0093] The top two results of BLAST searches with the native aad-12 nucleotide sequence show a reasonable level of homology (about 85%) over 120 basepairs of sequence. Hybridization under certain conditions could be expected to include these two sequences. *See* GENBANK Acc. Nos. DQ406818.1 (89329742; *Rhodoferax*) and AJ6288601.1 (44903451; *Sphingomonas*). *Rhodoferax* is very similar to *Delftia* but *Sphingomonas* is an entirely different Class phylogenetically.

[0094] The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[0095] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused

fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[0096] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[0097] Specific changes to the "active site" of the enzyme can be made to affect the inherent functionallity with respect to activity or stereospecificity (see alignment **Figure 2**). Muller *et. al.* (2006). The known tauD crystal structure was used as a model dioxygenase to determine active site residues while bound to its inherent substrate taurine. Elkins *et al.* (2002) "X-ray crystal structure of *Escerichia coli* taurine/alpha-ketoglutarate dioxygenase complexed to ferrous iron and substrates," *Biochemistry* 41(16):5185-5192. Regarding sequence optimization and designability of enzyme active sites, *see* Chakrabarti *et al.*, PNAS, (Aug. 23, 2005), 102(34):12035-12040.

[0098] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190,

191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, or 293 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[0099] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[00100] It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.*, Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term "protein" can include functionally active truncations.

[00101] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically

encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[00102] Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[00103] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.*, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[00104] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby

an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 2** provides a listing of examples of amino acids belonging to each class.

Table 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[00105] In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

[00106] As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

[00107] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

[00108] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or

monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

[00109] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf, legume forages (e.g., alafalfa and clover), pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[00110] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[00111] Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[00112] Although plants can be preferred, the subject invention also includes production of highly active recombinant AAD-12 in a *Pseudomonas fluorescens* (Pf) host strain, for example. The subject invention includes preferred growth temperatures for maintaining soluble active AAD-12 in this host; a fermentation condition where AAD-12 is produced as more than 40% total cell protein, or at least 10 g/L; a purification process results high recovery of active recombinant AAD-12 from a Pf host; a purification scheme which yields at least 10 g active AAD-12 per kg of cells; a purification scheme which can yield 20 g active

AAD-12 per kg of cells; a formulation process that can store and restore AAD-12 activity in solution; and a lyophilization process that can retain AAD-12 activity for long-term storage and shelf life.

[00113] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[00114] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[00115] Vectors comprising an *AAD-12* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc.* Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli.* The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

[00116] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle

bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria.* The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria.* They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[00117] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00118] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[00119] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate (*e.g., PAT/bar*), glyphosate (*EPSPS*), ALS-inhibitors (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, *et al.*), bromoxynil, HPPD-inhibitor resistance, PPO-inhibitors, ACC-ase inhibitors , and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00120] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. *See* U.S. Patent No. 6,809,232. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to

Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00121] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[00122] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00123] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation

thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

[00124] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00125] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G41;, hygromycin resistance; methotrexate resistance, as well as those genes which encode for resistance or tolerance to glyphosate; phosphinothricin (bialaphos or glufosinate); ALS-inhibiting herbicides (imidazolinones, sulfonylureas and triazolopyrimidine herbicides), ACC-ase inhibitors (e.g., ayryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

[00126] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-

glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00127] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00128] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such

include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00129] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00130] Means of further increasing tolerance or resistance levels. It is shown herein that plants of the subject invention can be imparted with novel herbicide resistance traits without observable adverse effects on phenotype including yield. Such plants are within the scope of the subject invention. Plants exemplified and suggested herein can withstand 2X, 3X, 4X, and 5X typical application levels, for example, of at least one subject herbicide. Improvements in these tolerance levels are within the scope of this invention. For example, various techniques are know in the art, and can forseeably be optimized and further developed, for increasing expression of a given gene.

[00131] One such method includes increasing the copy number of the subject AAD-12 genes (in expression cassettes and the like). Transformation events can also be selected for those having multiple copies of the genes.

[00132] Strong promoters and enhancers can be used to "supercharge" expression. Examples of such promoters include the preferred 35T promoter which uses 35S enhancers. 35S, maize ubiquitin, Arabidopsis ubiquitin, A.t. actin, and CSMV promoters are included for such uses. Other strong viral promoters are also preferred. Enhancers include 4 OCS and the 35S double enhancer. Matrix attachment regions (MARs) can also be used to increase transformation efficiencies and transgene expression, for example.

[00133] Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

[00134] Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See *e.g.* U.S. Patent No. 7,058,515; Larson et al., *Protein Sci.* 2002 11: 2804-2813, "Thoroughly sampling sequence space: Large-scale protein design of structural ensembles."; Crameri *et al.*, *Nature Biotechnology* 15, 436 - 438 (1997), "Molecular evolution of an arsenate detoxification pathway by DNA shuffling."; Stemmer, W.P.C.1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc. Natl. Acad. Sci. USA 91: 10747–10751; Stemmer, W.P.C.1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature 370: 389–391; Stemmer, W.P.C.1995. Searching sequence space. Bio/Technology 13: 549–553; Crameri, A., Cwirla, S. and Stemmer, W.P.C.1996. Construction and evolution of antibody-phage libraries by DNA shuffling. Nature Medicine 2: 100–103; and Crameri, A., Whitehorn, E.A., Tate, E. and Stemmer, W.P.C. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnology 14: 315–319.

[00135] The activity of recombinant polynucleotides inserted into plant cells can be dependent upon the influence of endogenous plant DNA adjacent the insert. Thus, another option is taking advantage of events that are known to be excellent locations in a plant genome for insertions. *See e.g.* WO 2005/103266 A1, relating to cry1F and cry1Ac cotton events; the subject AAD-12 gene can be substituted in those genomic loci in place of the cry1F and/or cry1Ac inserts. Thus, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (USPA 20030232410), relating to the use of zinc fingers for targeted recombination. The use of recombinases (cre-lox and flp-frt for example) is also known in the art.

[00136] AAD-12 detoxification is believed to occur in the cytoplasm. Thus, means for further stabilizing this protein and mRNAs (including blocking mRNA degradation) are included in aspects of the subject invention, and art-known techniques can be applied accordingly. The subject proteins can be designed to resist degradation by proteases and the like (protease cleavage sites can be effectively removed by re-engineering the amino acid sequence of the protein). Such embodiments include the use of 5' and 3' stem loop structures like UTRs

from osmotin, and per5 (AU-rich untranslated 5' sequences). 5' caps like 7- methyl or 2'-O-methyl groups, e.g., 7-methylguanylic acid residue, can also be used. *See, e.g.,*: Proc. Natl. Acad. Sci. USA Vol. 74, No. 7, pp. 2734-2738 (July 1977) *Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis.* Protein complexes or ligand blocking groups can also be used.

[00137] Computational design of 5' or 3' UTR most suitable for AAD-12 (synthetic hairpins) can also be conducted within the scope of the subject invention. Computer modeling in general, as well as gene shuffling and directed evolution, are discussed elsewhere herein. More specifically regarding computer modeling and UTRs, computer modeling techniques for use in predicting/evaluating 5' and 3' UTR derivatives of the present invention include, but are not limited to: MFold version 3.1 available from Genetics Corporation Group, Madison, WI (see Zucker et al., Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In *RNA Biochemistry and Biotechnology*, 11-43, J. Barciszewski & B.F.C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, NL, (1999); Zucker *et al.*, *Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure. J. Mol. Biol.* 288, 911-940 (1999); Zucker *et al.*, RNA Secondary Structure Prediction.In *Current Protocols in Nucleic Acid Chemistry* S. Beaucage, D.E. Bergstrom, G.D. Glick, and R.A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, (2000)), COVE (RNA structure analysis using covariance models (stochastic context free grammar methods)) v.2.4.2 (Eddy & Durbin, Nucl. Acids Res. 1994, 22: 2079-2088) which is freely distributed as source code and which can be downloaded by accessing the website genetics.wustl.edu/eddy/software/, and FOLDALIGN, also freely distributed and available for downloading at the website bioinf.au.dk. FOLDALIGN/ (see *Finding the most significant common sequence and structure motifs in a set of RNA sequences.* J. Gorodkin, L. J. Heyer and G. D. Stormo. Nucleic Acids Research, Vol. 25, no. 18 pp 3724-3732, 1997; *Finding Common Sequence and Structure Motifs in a set of RNA Sequences.*. J. Gorodkin, L. J. Heyer, and G. D. Stormo. ISMB 5;120-123, 1997).

[00138] Embodiments of the subject invention can be used in conjunction with naturally evolved or chemically induced mutants (mutants can be selected by screening techniques, then transformed with AAD-12 and possibly other genes). Plants of the subject invention can be combined with ALS resistance and/or evolved glyphosate resistance. Aminopyralid resistance, for example, can also be combined or "stacked" with an AAD-12 gene.

[00139] Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve desired traits.

[00140] Further improvements also include use with appropriate safeners to further protect plants and/or to add cross resistance to more herbicides. (Safeners typically act to increase plants immune system by activating/expressing cP450. Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism, without compromising weed control efficacy.)

[00141] Herbicide safeners include benoxacor, cloquintocet, cyometrinil, dichlormid, dicyclonon, dietholate, fenchlorazole, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen, mefenpyr, mephenate, naphthalic anhydride, and oxabetrinil. Plant activators (a new class of compounds that protect plants by activating their defense mechanisms) can also be used in embodiments of the subject invention. These include acibenzolar and probenazole.

[00142] Commercialized safeners can be used for the protection of large-seeded grass crops, such as corn, grain sorghum, and wet-sown rice, against preplant-incorporated or preemergence-applied herbicides of the thiocarbamate and chloroacetanilide families. Safeners also have been developed to protect winter cereal crops such as wheat against postemergence applications of aryloxyphenoxypropionate and sulfonylurea herbicides. The use of safeners for the protection of corn and rice against sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides is also well-established. A safener-induced enhancement of herbicide detoxification in safened plants is widely accepted as the major mechanism involved in safener action. Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione *S*-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. Hatzios KK, Burgos N (2004) "Metabolism-based herbicide resistance: regulation by safeners," Weed Science: Vol. 52, No. 3 pp. 454–467.

[00143] Use of a cytochrome p450 monooxygenase gene stacked with AAD-12 is one preferred embodiment. There are P450s involved in herbicide metabolism; cP450 can be of mammalian or plant origin, for example. In higher plants, cytochrome P450 monooxygenase (P450) is known to conduct secondary metabolism. It also plays an important role in the oxidative metabolism of xenobiotics in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase). Resistance to some herbicides has been reported as a result of the metabolism by P450 as well as glutathione S-transferase. A number of microsomal P450

species involved in xenobiotic metabolism in mammals have been characterized by molecular cloning. Some of them were reported to metabolize several herbicides efficiently. Thus, transgenic plants with plant or mammalian P450 can show resistance to several herbicides.

[00144] One preferred embodiment of the foregoing is the use cP450 for resistance to acetochlor (acetochlor-based products include Surpass®, Keystone®, Keystone LA, FulTime® and TopNotch® herbicides) and/or trifluralin (such as Treflan®). Such resistance in soybeans and/or corn is included in some preferred embodiments. For additional guidance regarding such embodiments, *see e.g.* Inui *et al.*, "A selectable marker using cytochrome P450 monooxygenases for Arabidopsis transformation," *Plant Biotechnology* 22, 281–286 (2005) (relating to a selection system for transformation of Arabidopsis thaliana via Agrobacterium tumefaciens that uses human cytochrome P450 monooxygenases that metabolize herbicides; herbicide tolerant seedlings were transformed and selected with the herbicides acetochlor, amiprophos-methyl, chlorpropham, chlorsulfuron, norflurazon, and pendimethalin); Siminszky et al., "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides," *PNAS* Vol. 96, Issue 4, 1750-1755, February 16, 1999; Sheldon et al, *Weed Science*: Vol. 48, No. 3, pp. 291–295, "A cytochrome P450 monooxygenase cDNA (CYP71A10) confers resistance to linuron in transgenic Nicotiana tabacum"; and "Phytoremediation of the herbicides atrazine and metolachlor by transgenic rice plants expressing human CYP1A1, CYP2B6, and CYP2C19," *J Agric Food Chem.* 2006 Apr 19;54(8):2985-91 (relating to testing a human cytochrome p450 monooxygenase in rice where the rice plants reportedly showed high tolerance to chloroacetomides (acetochlor, alachlor, metoachlor, pretilachlor, and thenylchlor), oxyacetamides (mefenacet), pyridazinones (norflurazon), 2,6-dinitroanalines (trifluralin and pendimethalin), phosphamidates (amiprofos-methyl, thiocarbamates (pyributicarb), and ureas (chlortoluron)).

[00145] There is also the possibility of altering or using different 2,4-D chemistries to make the subject AAD-12 genes more efficient. Such possible changes include creating better substrates and better leaving groups (higher electronegativity).

[00146] Auxin transport inhibitors (e.g. diflufenzopyr) can also be used to increase herbicide activity with 2,4-D.

[00147] Unless specifically indicated or implied, the terms "a", "an", and "the" signify "at least one" as used herein.

[00148] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00149] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Method for Identifying Genes That Impart Resistance to 2,4-D *In Planta*

[00150] As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (*i.e.*, α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with ≤ 50% homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to *tfdA* from *Ralstonia eutropha)* can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00151] A single gene (*sdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 31% amino acid identity to *tfdA*. Percent identity was determined by first translating both the *sdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

Example 2 – Optimization of Sequence for Expression in Plants and Bacteria

[00152] 2.1 – Background.

[00153] To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[00154] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00155] **Table 3** illustrates how high the G+C content is in maize. For the data in **Table 3**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

Table 3: Compilation of G + C contents of protein coding regions of maize genes

Protein Class[a]	Range % G + C	Mean % G + C[b]
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)[c]

[a] Number of genes in class given in parentheses.
[b]Standard deviations given in parentheses.
[c] Combined groups mean ignored in mean calculation

[00156] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[00157] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 4**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new

sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, *etc.* with the next preferred codon of choice.

Table 4. Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

[00158] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and

about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00159] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

[00160] 2.2 – *AAD-12* Plant rebuild analysis.

[00161] Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native *AAD-12* coding region (SEQ ID NO:1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 (*AAD-12*) is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence *AAD-12 (v1)* (SEQ ID NO:3) was developed that encodes a protein (SEQ ID NO:4) which is the same as the native SEQ ID NO:2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO:4). The additional alanine codon (GCT; underlined in SEQ ID NO:3) encodes part of an *NcoI* restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical. **Table 5** shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences

(Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

Table 5. Codon composition comparisons of coding regions of Native *AAD-12*, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #	Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #
ALA (A)	GCA	1	10	11	LEU (L)	CTA	0	0	0
	GCC	35	16	15		CTC	1	8	8
	GCG	7	0	0		CTG	23	0	0
	GCT	0	18	17		CTT	0	8	8
ARG (R)	AGA	0	4	5		TTA	0	0	0
	AGG	0	4	6		TTG	0	8	8
	CGA	0	0	0	LYS (K)	AAA	1	1	2
	CGC	15	6	4		AAG	5	5	4
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	4	3	PHE (F)	TTC	7	5	5
ASN (N)	AAC	3	2	2		TTT	1	3	3
	AAT	1	2	2	PRO (P)	CCA	0	5	6
ASP (D)	GAC	15	9	9		CCC	9	4	4
	GAT	2	8	8		CCG	5	0	0
CYS (C)	TGC	3	2	2		CCT	0	5	5
	TGT	0	1	1	SER (S)	AGC	5	4	3
END	TAA	1	0	1		AGT	0	0	0
	TAG	0	0			TCA	0	3	3
	TGA	0	1			TCC	2	3	3
GLN (Q)	CAA	1	8	7		TCG	6	0	0
	CAG	13	6	7		TCT	0	3	3
GLU (E)	GAA	3	4	4	THR (T)	ACA	1	4	5
	GAG	8	7	7		ACC	11	7	7
GLY (G)	GGA	0	8	7		ACG	5	0	0
	GGC	24	7	7		ACT	1	7	6
	GGG	1	3	4	TRP (W)	TGG	8	8	8
	GGT	0	7	7	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	9	9		TAT	1	2	2
	CAT	8	7	7	VAL (V)	GTA	0	0	0
ILE (I)	ATA	0	2	2		GTC	6	8	7
	ATC	10	4	5		GTG	18	8	9
	ATT	1	5	4		GTT	0	8	8
	Totals	163	164	163		Totals	130	130	130

[00162] It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

[00163] 2.3 Rebuild for *E. coli* Expression

[00164] Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in *Escherichia coli and Salmonella typhimurium cellular and molecular biology, vol. 2*, pp. 2047–2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umbarger, H. (eds.) American Society for Microbiology, Washington, DC). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for these *E. coli*-Class II gene coding regions. It is thought that a protein coding region having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO:4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (*i.e.* Shine Dalgarno sequences) The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO:5 and encodes the protein disclosed in SEQ ID NO:4.

[00165] The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. Table 6 presents the codon compositions of the native *AAD-12* coding region (Columns A and D), an *AAD-12* coding region optimized for expression in *E. coli* (v2; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).

Table 6. Codon composition comparisons of coding regions of Native *AAD-12*, *E. coli*-Optimized version (v2) and a Theoretical *E. coli* Class II-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor. Class II #	Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor, Class II #
ALA (A)	GCA	1	13	13	LEU (L)	CTA	0	0	0
	GCC	35	0	0		CTC	1	2	0
	GCG	7	18	17		CTG	23	20	24
	GCT	0	13	14		CTT	0	1	0
ARG (R)	AGA	0	0	0		TTA	0	1	0
	AGG	0	0	0		TTG	0	0	0
	CGA	0	0	0	LYS (K)	AAA	1	4	5
	CGC	15	6	6		AAG	5	2	1
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	12	12	PHE (F)	TTC	7	6	6
ASN (N)	AAC	3	4	4		TTT	1	2	2
	AAT	1	0	0	PRO (P)	CCA	0	3	2
ASP (D)	GAC	15	10	9		CCC	9	0	0
	GAT	2	7	8		CCG	5	11	12
CYS (C)	TGC	3	2	2		CCT	0	0	0
	TGT	0	1	1	SER (S)	AGC	5	4	4
END	TAA	1	1	1		AGT	0	0	0
	TAG	0	0	0		TCA	0	0	0
	TGA	0	0	0		TCC	2	5	4
GLN (Q)	CAA	1	3	3		TCG	6	0	0
	CAG	13	11	11		TCT	0	4	5
GLU (E)	GAA	3	8	8	THR (T)	ACA	1	0	0
	GAG	8	3	3		ACC	11	12	12
GLY (G)	GGA	0	0	0		ACG	5	0	0
	GGC	24	12	11		ACT	1	6	6
	GGG	1	0	0	TRP (W)	TGG	8	8	8
	GGT	0	13	14	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	11	11		TAT	1	2	2
	CAT	8	5	5	VAL (V)	GTA	0	6	6
ILE (I)	ATA	0	0	0		GTC	6	0	0
	ATC	10	7	7		GTG	18	8	7
	ATT	1	4	4		GTT	0	10	11
	Totals	163	164	164		Totals	130	130	130

[00166] It is clear from examination of Table 6 that the native and *E. coli*-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

[00167] 2.4 – Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate

synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO:20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00168] A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 7. Columns D and H of Table 7 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 7. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

Weighted % of C1 =1/(%C1 + %C2 + %C3 + *etc.*) x %C1 x 100

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 7 (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of Table 7. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table

for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

Table 7. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	**Codon**	**Weighted %**	**Soybean %**	**Amino Acid**	**Codon**	**Weighted %**	**Soybean %**
ALA (A)	GCA	33.1	30.3	**LEU (L)**	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	**LYS (K)**	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	**6.0**	**MET (M)**	ATG	100.0	100
	CGT	16.9	14.5	**PHE (F)**	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	**PRO (P)**	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	**SER (S)**	AGC	16.0	15.1
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	**6.1**
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	**THR (T)**	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	**8.3**
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	**TRP (W)**	TGG	100.0	100
	GGT	30.4	30.4	**TYR (Y)**	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	**VAL (V)**	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

[00169] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 from PCT/US2005/014737 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00170] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is disclosed as bases 1-1575 of SEQ ID NO:22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO:22 of PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston TX).

Example 3-Cloning of expression and transformation vectors

[00171] 3.1 Construction of E.coli, pET expression vector.

[00172] Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba 1, Xho 1) *AAD-12 (v2)* was cut out of the picoscript vector, and ligated into a pET280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

[00173] To differentiate between *AAD-12 (v2)* : pET280 and pCR2.1 : pET280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB-S/S, and grown at 37° C for 4 hours with agitation. Each culture was then spotted onto LB + Kanamycin 50 μg/ml plates, which were incubated at 37° C overnight. Colonies that grew on the LB-K were assumed to

have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before, and checked for correctness with digestion by XbaI / XhoI. The final expression construct was given the designation pDAB3222.

[00174] 3.2- Construction of *Pseudomonas* expression vector

[00175] The *AAD-12 (v2)* open reading frame was initially cloned into the modified pET expression vector (Novagen), "pET280 S/S", as an XbaI – XhoI fragment. The resulting plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The *AAD-12 (v2)* open reading frame from pDAB725 was transferred into the *Pseudomonas* expression vector, pMYC1803, as an XbaI – XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB324 *Pseudomonas* expression strains.

[00176] 3.3 – Completion of binary vectors.

[00177] The plant optimized gene *AAD-12 (v1)* was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:6) and M13 Reverse (SEQ ID NO:7) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-12 (v1)* DNA sequence. The *AAD-12 (v1)* gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-12 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB724, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-12 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium* (see section 7.2).

[00178] 3.4 - Cloning of additional transformation constructs.

[00179] All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular

cloning methods (Maniatis *et al.,* 1982). **Table 8** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

Table 8. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
724	-	A, Ct, S	AAD12 v1	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3274	-	A	AAD12 v1	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	-	-	Agro binary
3278	1580	T	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2			Spectinomycin	-	pat	AtUbi10	Agro binary
3285	-	A	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3759	-	A, Ca, S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi10	Agro binary
4101	1863	Cn, R	AAD12 v1	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers / Gun
4464	-	S	AAD12 v1	CsVMV	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4468	-	S	AAD12 v1	AtUbi10	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4472	-	S	AAD12 v1	AtUbi3	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4476	-	S	AAD12 v1	ZmUbi1	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4480	-	S	AAD12 v1	AtAct2	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary

*A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
Atubi3= *Arabidopsis thaliana* Ubiquitin 3 Promoter
AtAct2= *Arabidopsis thaliana* Actin 2 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

Example 4 - Recombinant AAD-12 (v2) Expression and Purification in *Pseudomonas fluorescens*

[00180] 4.1- *Pseudomonas fluorescens* fermentation

[00181] For shake flask experiment, 200 µl of *Pseudomonas fluorescens* strain MB324 glycerol stock carried *AAD-12* (v2) construct pDAB739 (sec 3.2) was used to inoculate 50 ml fresh LB media supplemented with 30 µg/ml tetracycline/HCl. The culture (in a 250 ml baffled Erlenmeyer flask) was incubated on a shaker (New Brunswick Scientific Model Innova 44) at 300 rpm and 30 °C for 16 hrs. 20 ml of seed culture was transferred into 1 L *Pseudomonas fluorescens* culture media (Yeast extract, 5 g/L; K_2HPO_4, 5 g/L; $(NH_4)_2PO_4$, 7.5 g/L; $(NH_4)_2SO_4$; $MgSO_4$-$7H_2O$, 1 g/L; KCl, 0.5 g/L; $CaCl_2$-$2H_2O$, 0.5 g/L; NaCitrate-$2H_2O$, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: $FeCl_3$-$6H_2O$, 5.4 g/L; $MnCl_2$-$4H_2O$, 1 g/L; $ZnSO_4$-$7H_2O$, 1.45 g/L; $CuSO_4$-$5H_2O$, 0.25 g/L; H_3BO_3, 0.1 g/L; $(NH_4)_6MO_7O_{24}$, 0.1 g/L; concentrated HCl, 13 ml/L) supplemented with 20 µg/ml tetracycline/HCl and 250 µl of Pluronic L61(anti-foam) in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 30 °C and 300 rpm for 24 hrs. Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was added to 1 mM final in the cultures and continued to incubate for approximately 48 hrs at 25 °C. Cells were harvested by centrifugation at 7 krpm at 4 °C for 15 min, and cell paste was stored at -80 °C or immediately processed for purification.

[00182] For tank experiments, 1 ml each of the glycerol stock was inoculated a 1 L baffled flask containing 200 ml of LB media supplemented with 30 µg/ml tetracycline/HCl at 300 rpm and 32 °C for 16-24 hrs. The combined culture from three flasks (600 ml) was then aseptically transferred to a 20 L fermentor (B. Braun Bioreactor Systems) containing 10 L of Dow proprietary defined medium (through Teknova, Hollister, CA) designed to support high cell density growth. Growth temperature was maintained at 32 °C and the pH was controlled at the desired set-point through the addition of aqueous ammonia. Dissolved oxygen was maintained at a positive level in the liquid culture by regulating the sparged air flow and the agitation rates. The fed-batch fermentation process was carried out for approximately 24 hrs till cell density reached 170-200 OD_{575}. IPTG was added to 1 mM to induce the recombinant protein expression and the temperature was reduced and maintained to 25 °C using circulation of cold-water supply. The induction phase of the fermentation was allowed to continue for another 24 hrs. Samples (30 ml) were collected for various analyses to determine cell density

and protein expression level at 6, 12, and 18 hrs post-induction time points. At the end of a fermentation run, cells were harvested by centrifugation at 10 krpm for 30 min. The cell pellets were frozen at -80 °C for further processing.

[00183] 4.2- Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

[00184] Approximately 100-200 g of frozen (or fresh) *Pseudomonas* cells were thawed and resuspended in 1-2 L of extraction buffer containing 20 mM Tris-HCl, pH 8.5, and 25 ml of Protease inhibitor cocktail (Sigma cat# P8465). The cells were disrupted using Microfluidizer (model M110L or 110Y) (Microfluidics, Newton, MA) on ice with one pass at 11,000-12,000 psi. The lysate was centrifuged at 24,000 rpm for 20 min. The supernatant was transferred and dialyzed against 10 volumes of 20 mM Tris-HCl, pH 8.5 overnight at 4 °C, or diafiltrated with this buffer and filtered through a 0.45 μm membrane before applying to the column separations. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4 °C. Prior to loading, a Q Sepharose Fast Flow column (Pharmacia XK 50/00, 500 ml bed size) was equilibrated with 20 mM Tris-HCl, pH 8.5 buffer. The sample was applied to the column at 15 ml/min and then washed with this buffer until the eluate OD_{280} returned to baseline. Proteins were eluted with 2 L of linear gradient from 0 to 0.3 M NaCl at a flow rate of 15 ml/min, while 45 ml fractions were collected. Fractions containing AAD-12 activity as determined by the colorimetric enzyme assay and also corresponding to the predicted molecular weight of AAD-12 protein (about 32 kDa band on SDS-PAGE) were pooled. Solid ammonium sulfate to final 0.5 M was added to the sample, and then applied to a Phenyl HP column (Pharmacia XK 50/20, 250 ml bed size) equilibrated in 0.5 M ammonium sulfate in 20 mM Tris-HCl, pH 8.0. This column was washed with the binding buffer at 10 ml/min until the OD_{280} of the eluate returned to baseline, proteins were eluted within 2 column volumes at 10 ml/min by a linear gradient from 0.5 M to 0 Ammonium sulfate in 20 mM Tris-HCl, pH 8.0, and 12.5 ml fractions were collected. The main peak fractions containing AAD-12 were pooled, and if necessary, concentrated using a MWCO 10 kDa cut-off membrane centrifugal filter device (Millipore). In some cases the sample was further applied to a Superdex 75 gel filtration column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure AAD-12 were pooled and stored at -80 °C. In most cases, AAD-12 protein purity is approaching or above 99% after sequential ion-exchange column and

hydrophobic interaction column two-step separation. A typical yield for purified AAD-12 is 12-18 mg/g of wet cells. Bulk protein sample was formulated in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM DTT, and 1% Trehalose by diafiltration, and lyophilized on the Virtis Freezemobile Model 25EL (Virtis, Cardiner, NY) for long-term storage.

[00185] Protein concentration was initially measured by Bradford assay using Bio-Rad Protein assay kit (cat# 500-0006) with bovine serum albumin as standard. When needed, more accurate protein concentration was determined by using total amino acid hydrolysis. The sample was analyzed in Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) with amino acid calibration standards (cat# PN5061-3330) purchased from Agilent.

[00186] AAD-12 activity was determined through out the processes to ensure no loss of the enzyme activity by each treatment and manipulation, as described in the Example 5 below. Protein purity was monitored by using SDS-PAGE and analytical size exclusion chromatography. Purified protein sample was further verified and confirmed by N-terminal amino acid sequencing, and shown consisting of expected AQTTLQITPT residues at its N-terminus. Short and long-term protein stability was tested by enzymatic activity and by native-PAGE and SDS-PAGE gel analysis under both non-reducing and reducing conditions. And it was noticed that AAD-12 is prone to oligomerization via disulfide bond formation, therefore typically 2 mM DTT was used for protein storage. Phosphate-buffer saline (PBS) and Tris-buffer saline (TBS) were tested for protein lyophilization, with and without the presence of 1% trehalose. Additionally, the endotoxin and DNA contaminant context from purified sample were measured respectively, and the integrity of the AAD-12 protein was also assessed by isoelectric focusing (IEF) analysis.

[00187] Ten milligrams of purified AAD-12 (v2) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 ml of complete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification, and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

Example 5 – *In vitro* assays of AAD-12 activity

[00188] 5.1 – Assay via colorimetric phenol detection.

[00189] Enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2 μM phenol.

[00190] Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 5 minutes of incubation at 25° C, the reaction was terminated by addition of 30 μl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH) and 0.2 % 4-aminoantipyrine. Then 10 μl 0.8 % potassium ferricyanide was added and after 5 or 10 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols.

[00191] 5.2 – Assay via detection of chloropyridinol

[00192] AAD-12 action on potential substrates such as the herbicide triclopyr containing a substituted pyridine (rather than benzene rings) will release a pyridinol on cleavage of the aryloxyalkanoate bond. Pyridinols were not detected using the aminoantipyrine/ferricyanide phenol detection described in the preceding section. However, it was found that product chloropyridinols absorb strongly in the near UV with λ_{max} of 325 nm at pH 7 (extinction coefficient ~8,400 $M^{-1}.cm^{-1}$). This was used to create a continuous microplate-based spectrophotometric assay. Assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate,

the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero and the increase in absorbance followed for 10 minutes at 325 nm in a microplate reader. The first 2 minutes of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 µl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9 µM chloropyridinol.

[00193] 5.3 – Colorimetric assay using 2-(2-chloro,4-nitrophenoxy)propionate

[00194] A convenient assay of AAD-12 was devised using 2-(2-chloro,4-nitrophenoxy)propionate (CNPP) as substrate. Cleavage of CNPP by AAD-12 releases 2-chloro,4-nitrophenol. This phenol has a bright yellow absorbance at 410 nm at pH 7 enabling the reaction to be followed continuously or by endpoint analysis. The presence of AAD-12 activity can be monitored visually without the need for addition of further reagents. Microplate-based spectrophotometric assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 µM NH_4FeSO_4, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate amount of CNPP (added from a 10 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of CNPP, enzyme, or α-ketoglutarate at time zero and the increase in absorbance followed for 10 min at 410 nm in a microplate reader. The first 2 min of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 µl final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1 µM 2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be $K_m = 31 \pm 5.5$ µM and $k_{cat} = 16.2 \pm 0.79$ min^{-1}.

Example 6 – *In vitro* activity of AAD-12 on various substrates

[00195] 6.1 – AAD-12 (v2) activity on (R,S)- dichlorprop, (R)-dichlorprop, (S)-dichlorprop and 2,4-D

[00196] Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4 µg purified AAD-12 (v2). (R,S)-dichlorprop (R,S-DP) was tested at 1 mM and (R)-dichlorprop, (S)-dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in **Figure 3**, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4 µg AAD-12 (v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of α-

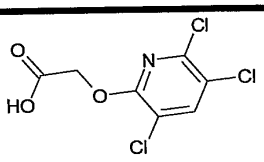
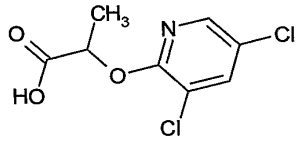
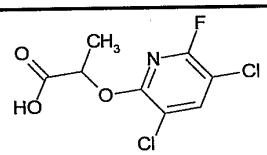
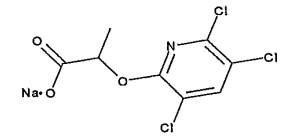
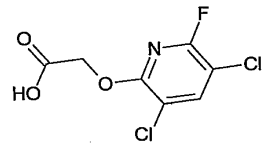
ketoglutarate. After 5 minutes, the reaction was quenched, and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

[00197] AAD-12 (v2) has excellent activity on (R,S)-dichlorprop and (S)-dichlorprop and has minimal activity on (R)-dichlorprop. This indicates that AAD-12 (v2) has a clear (S)-enantiomeric preference. The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)-dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively.

[00198] 6.2 – AAD-12 (v2) activity on pyridyloxyalkanoates

[00199] Using the pyridinol assay described in Example 5.2, five pyridyloxyalkanoates were assayed at 1 mM in a reaction mix containing 6.8 µg purified AAD-12 (v2). The rates of each reaction were monitored and are presented in Table 9. All five pyridyloxyalkanoates were cleaved to release pyridinols by AAD-12 (v2). The rates for the oxypropionate substrates 116844 and 91767 were somewhat faster than those for the corresponding acetates (triclopyr and 93833 respectively) indicating a preference of AAD-12 (v2) for oxypropionate over oxyacetate side chains. These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.

Table 9. Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8µg AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of α-ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without α-ketoglutarate was subtracted from the rates with substrate.

STRUCTURE	ID	Rate (mAU/min)	Rate relative to triclopyr
	triclopyr	97	1
	66357	225	2.3
	91767	190	0.8
	116844	257	1.4
	93833	118	0.5

[00200] 6.3 – Kinetic constants of AAD-12 (v2) for 2,4-D, (R,S)-DCP and triclopyr

[00201] The K_m and k_{cat} values of purified AAD-12 (v2) for the herbicides 2,4-D, (R,S)-dichlorprop and triclopyr were determined using the appropriate assay method. Substrate inhibition occurred at high concentrations (>1 mM) of 2,4-D and (R,S)-DCP so concentrations below this were used to fit the data to the Michaelis-Menten equation using

Grafit 4.0 (Erithacus Software, UK). No substrate inhibition was noted for triclopyr up to 2 mM. The kinetic constants are summarized in Table 10. From these data, the rate of AAD-12 (v2) cleavage of triclopyr is ~5% that of 2,4-D, under maximal velocity conditions.

Table 10. Kinetic constants of AAD-12 (v2) for three herbicide substrates

Substrate	K_m, μM (±SE)	k_{cat}, min^{-1} (±SE)	Assay method	Substrate inhibition at 2 mM
2,4-D	102 (±18.4)	54.1 (±3.1)	Phenol detection	55%
(R,S)-dichlorprop	122 (±2.7)*	63.4 (±0.5)	Phenol detection	55%
Triclopyr	241 (±30)	2.6 (±0.1)	ΔA325 nm	0%

* Because of the (S)-enantiomeric preference of AAD-12, the K_m value was calculated assuming 50% of the racemic mixture was available as a substrate

Example 7 – Transformation into *Arabidopsis* and Selection

[00202] 7.1 – *Arabidopsis thaliana* growth conditions.

[00203] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00204] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00205] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

[00206] 7.2 – *Agrobacterium* transformation.

[00207] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA)

Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

[00208] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 µl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00209] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00210] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed Agrobacterium cells, suspended in 10 µl of water, at 100° C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by

ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

[00211] 7.3 – *Arabidopsis* transformation.

[00212] *Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044µM benzylamino purine (10 µl/liter of 1 mg/ml stock in DMSO) and 300 µl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

[00213] 7.4 – Selection of transformed plants.

[00214] Freshly harvested T_1 seed [*AAD-12 (v1)* gene] was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00215] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00216] Seven days after planting (DAP) and again 11 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-12 (v1)* (plant optimized gene) to provide phenoxy auxin herbicide resistance.

[00217] T_1 plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis,* 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200g ae/ha). Tables 10 and 11 show comparisons drawn to an aryloxyalkanoate herbicide resistance gene *(AAD-1 (v3))* previously described in PCT/US2005/014737.

[00218] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

[00219] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluroxypyr, could be effectively degraded *in planta*. Herbicides

were applied to T_1 plants with use of a track sparyer in a 187 L/ha spray volume. T_1 plants that exhibited tolerance to 2,4-D DMA were further accessed in the T_2 generation.

[00220] 7.5 – Results of selection of transformed plants.

[00221] The first *Arabidopsis* transformations were conducted using *AAD-12 (v1)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T_1 seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT + Liberty are used for selection. T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 11 compares the response of *AAD-12 (v1)* and control genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The *AAD-12 (v1)* gene imparted herbicide resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 11 simply to demonstrate the significant difference between the plants transformed with *AAD-12 (v1)* versus the wildtype or *PAT/Cry1F*-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or 6x field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

Table 11. *AAD-12 (v1)* transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence, compared to an *AAD-1 v3* (T_4) homozygous resistant population, or a *Pat-Cry1F* transformed, auxin-sensitive control.

AAD-12 (v1) gene T_1 transformants Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control-buffer	6	0	0	0	0
50 g ae/ha 2,4-D	6	0	2	16	24
200 g ae/ha 2,4-D	6	1	1	11	18
800 g ae/ha 2,4-D	5	2	1	15	20
3200 g ae/ha 2,4-D	8	0	0	6	6
PAT/Cry1F (transformed control) Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	4	1	5	31	16
200 g ae/ha 2,4-D	0	0	10	70	2
800 g ae/ha 2,4-D	0	0	10	81	8
3200 g ae/ha 2,4-D	0	0	10	91	2
Homozygous AAD-1 (v3) gene T_4 plants Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	10	0	0	0	0
200 g ae/ha 2,4-D	10	0	0	0	0
800 g ae/ha 2,4-D	10	0	0	0	0
3200g ae/ha 2,4-D	9	1	0	2	6

[00222] Table 12 shows a similarly conducted dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. The data shows that the herbicidally active (R-) isomer of dichlorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-dichlorprop well enough to impart commercially acceptable tolerance is one distinguishing characteristic that separates the two genes. (Table 12). AAD-1 and AAD-12 are considered R- and S-specific α-ketoglutarate dioxygenases, respectively.

Table 12. T_1 *Arabidopsis* response to a range of R-dichlorprop rates applied postemergence.

AAD-12 v1 gene Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control	6	0	0	0	0
50 g ae/ha R-dichlorprop	0	0	8	63	7
200 g ae/ha R-dichlorprop	0	0	8	85	10
800 g ae/ha R-dichlorprop	0	0	8	96	4
3200 g ae/ha R-dichlorprop	0	0	8	98	2

PAT/Cry1F Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	0	10	0	27	2
200 g ae/ha R-dichlorprop	0	0	10	69	3
800 g ae/ha R-dichlorprop	0	0	10	83	6
3200 g ae/ha R-dichlorprop	0	0	10	90	2

Homozygous AAD-1 (v3) gene T_4 plants	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	10	0	0	0	0
50g ae/ha R-dichlorprop	10	0	0	0	0
200 g ae/ha R-dichlorprop	10	0	0	0	0
800 g ae/ha R-dichlorprop	10	0	0	0	0
3200 g ae/ha R-dichlorprop	10	0	0	0	0

[00223] 7.6 – *AAD-12 (v1)* as a selectable marker.

[00224] The ability to use *AAD-12 (v1)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed as described above. Approximately 50 T_4 generation *Arabidopsis* seed (homozygous for *A AD-12 (v1)*) were spiked into approximately 5,000 wildtype (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 follwed by 11 DAP. Since all individuals also contained the *PAT* gene in the same transformation vector, *AAD-12* selected with 2,4-D could be directly compared to *PAT* selected with glufosinate.

[00225] Treatments were applied with a DeVilbiss spray tip as previously describd. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate *AAD-12 (v1)* can be effectively used as an alternative selectable marker for a population of transformed *Arabidopsis*.

[00226] 7.7 – Heritability.

[00227] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). Seventy-five percent of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00228] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show *AAD-12 (v1)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

[00229] 7.8 – Additional foliar applications herbicide resistance in AAD-12 *Arabidopsis*.

[00230] The ability of *AAD-12 (v1)* to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates. T_2 generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing *PAT* and the insect resistance gene *Cry1F* was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Garlon 3A, Dow AgroSciences) and 280-2240 g ae/ha fluroxypyr (Starane, Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ae/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

[00231] There is no effect of the 2,4-D metabolite, 2,4-dichlorophenol (DCP), on transgenic non-*AAD-12* control *Arabidopsis (Pat/Cry1F)*. *AAD-12*-transformed plants were also clearly protected from the triclopyr and fluroxypyr herbicide injury that was seen in the transformed non-resistant conrols (see Table 13). These results confirm that *AAD-12 (v1)* in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. This is the first report of an enzyme with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

Table 13. Comparison of T_2 AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

	Pyridyloxyacetic auxins	
	Ave % Injury 14DAT	
Herbicide Treatment	**Segregating T_2 *AAD-12 (v1)* plants (pDAB724.01.120)**	**Pat/Cry1f - Control**
280 g ae/ha Triclopyr	0	52
560 g ae/ha Triclopyr	3	58
1120 g ae/ha Triclopyr	0	75*

Table 13. Comparison of T_2 AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.		
2240 g ae/ha Triclopyr	3	75*
280 g ae/ha Fluroxypyr	0	75*
560 g ae/ha Fluroxypyr	2	75*
1120 g ae/ha Fluroxypyr	3	75*
2240 g ae/ha Fluroxypyr	5	75*
	Inactive DCP metabolite	
280 g ae/ha 2,4-DCP	0	0
560 g ae/ha 2,4-DCP	0	0
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

* Plants in this experiment were stunted and severely epinastic, but remained green and did not receive injury ratings > 75%.

[00232] 7.9 – Molecular analysis of *AAD-12 (v1) Arabidopsis*.

[00233] Invader Assay (methods of Third Wave Agbio Kit Procedures) for *PAT* gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-12 (v1)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-12 (v1)*. Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00234] Results showed that all 2,4-D resistant plants assayed, contained *PAT* (and thus by inference, *AAD-12 (v1)*). Copy number analysis showed total inserts ranged from 1 to 5 copies. This correlates, too, with the *AAD-12 (v1)* protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

[00235] 7.10 – *Arabidopsis* transformed with molecular stack of *AAD-12 (v1)* and a glyphosate resistance gene.

[00236] T_1 *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (*AAD-12 (v1)* + *EPSPS*) which encodes a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-12 (v1)* as the selectable marker as described in example 7.6. T_1 plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-12 (v1)* + *PAT* T_4 homozygous lines (pDAB724-transformed), and *PAT* + *Cry1F* homozygous

line (transformed control). The pDAB3759 and pDAB724 transformed plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

[00237] Initial resistance assessment indicated plants tolerant to 2,4-D were subsequently tolerant to glyphosate when compared to the response of the three control lines. These results indicate that resistance can be imparted to plants to two herbicides with differening modes of action, including 2,4-D and glyphosate tolerance, allowing application of both herbicides postemergence. Additionally, AAD-12 + 2,4-D was used effectively as a selectable marker for a true resistance selection.

Table 14. T_1 *Arabidopsis* response to a range of glyphosate rates applied postemergence (14 DAT).

AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	13	2	1	11	16
105 g ae/ha glyphosate	10	1	5	34	38
420 g ae/ha glyphosate	5	6	5	44	37
1680 g ae/ha glyphosate	0	0	16	85	9

PAT/Cry1F Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	67	7
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

Wildtype (Col-0) Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	75	13
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

pDAB724 T_4 (PAT + AAD-12) Averages	% Injury <20%	20-40%	% Injury >40%	Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	66	8
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

[00238] 7.11 – *AAD-12 Arabidopsis* genetically stacked with *AAD-1* to give wider spectrum of herbicide tolerance.

[00239] *AAD-12 (v1)* (pDAB724) and *AAD-1 (v3)* (pDAB721) plants were reciprocally crossed and F_1 seed was collected. Eight F_1 seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F_1 plants and subjected to Western analysis to confirm the presence of both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

[00240] One hundred F_2 seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F_2 plants survived glufosinate selection fitting an expected segregation ration for two independently assorting loci for glufosinate resistance (15 R : 1S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop + 560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are consistent with an expected segregation pattern (9R: 6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlroprop or triclopyr). The results indicate that *AAD-12* (pDAB724) can be successfully stacked with *AAD-1* (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D + R-dichlorprop) and (2,4-D + fluroxypyr + triclopyr), respectively]. This could be useful to bring 2,4-D tolerance to a very sensitive species through conventional stacking of two separate 2,4-D resistance genes. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F_1 generation through paired sprays with herbicides that are exclusive between the AAD-1 and

AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively, above).

[00241] Other AAD stacks are also within the scope of the subject invention. The TfdA protein discussed elsewhere herein (Streber *et al.*), for example, can be used together with the subject AAD-12 genes to impart nover spectrums of herbicide resistance in transgenic plants of the subject invention.

Example 8 –WHISKERS-mediated transformation of corn using imazethapyr selection

[00242] 8.1 – Cloning of *AAD-12 (v1)*.

[00243] The *AAD-12 (v1)* gene was cut out of the intermediate vector pDAB3283 as an NcoI/SacI fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1 : *AAD-12 (v1)* cassette. This construct was digested with NotI and PvuI to liberate the gene cassette and digest the unwanted backbone. This was ligated to NotI cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101 or pDAS1863, and contains ZmUbi1/*AAD-12 (v1)*/ZmPer5::OsAct1/AHAS /LZmLip.

[00244] 8.2 – Callus/suspension initiation.

[00245] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00246] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was

selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00247] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

[00248] 8.3 – Cryopreservation and thawing of suspensions.

[00249] Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

[00250] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4)

in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 ml H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

[00251] 8.4 – Stable Transformation

[00252] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker shaken at 125 RPM in the dark for 30-35 minutes at 28°C, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume8.1 ml of GN6 S/M liquid medium to ~405 mg of pre-autoclaved,sterile silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00253] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml wasthen added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker at 125 RPM for 2 hours at 28°C before being filtered onto

Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00254] Approximately 2 ml of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box.

[00255] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 3 μM imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8). Plates were placed in boxes and cultured for an additional week.

[00256] Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 ml of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121°C) containing 3 μM imazethapyr from Pursuit® DG. The tissue was broken up and the 3 ml of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured until putative isolates appeared.

[00257] 8.4.1- Protocol for Isolate recovery and regeneration.

[00258] Putatively transformed events were isolated off the Pursuit®-containing embedded plates approximately 9 weeks post-transformation by transferring to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth was evident after approximately 2-3 weeks, the event was deemed to be resistant and was submitted for molecular analysis.

[00259] Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3 μM imazethapyr from Pursuit® DG, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week, then higher light (40 $\mu Em^{-2}s^{-1}$) for another week, before being transferred to regeneration medium, 36 (3P), which was identical to 28 (3P) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L

Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

[00260] From 4 experiments, full plantlets, comprised of a shoot and root, were formed *in vitro* on the embedded selection plates under dark conditions without undergoing a traditional callus phase. Leaf tissue from nine of these "early regenerators" were submitted for coding region PCR and Plant Transcription Unit (PTU) PCR for the *AAD-12* gene and gene cassette, respectively. All had an intact *AAD-12* coding region, while 3 did not have a full-length PTU (Table 15). These "early regenerators" were identified as 4101 events to differentiate them from the traditionally-derived events, which were identified as "1283" events.Plants from 19 additional events, obtained via standard selection and regeneration, were sent to the greenhouse, grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T_1 seed. Some of the events appear to be clones of one another due to similar banding patterns following Southern blot, so only 14 unique events were represented. T_0 plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (*AHAS* gene) indicated instertion complexity ranging from 1 to >10 copies. Thirteen events contained the compete coding region for *AAD-12*; however, further anaylsis indicated the complete plant transformation unit had not been incorporated for nine events. None of the compromised 1863 events were advanced beyond the T1 stage and further characterization utilized the 4101 events.

[00261] 8.5– Molecular Analysis: Maize Materials and Methods.

[00262] 8.5.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00263] 8.5.2 – Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl is placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well is overlaid with 15 µl

of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

[00264] 8.5.3 – Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-12 (v1)* PTU are Forward – GAACAGTTAGACATGGTCTAAAGG (SEQ ID NO:8) and Reverse – GCTGCAACACTGATAAATGCCAACTGG (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes.

[00265] Primers for *AAD-12 (v1)* Coding Region PCR are Forward – ATGGCTCAGACCACTCTCCAAA (SEQ ID NO:10) and Reverse – AGCTGCATCCATGCCAGGGA (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00266] 8.5.4 – Southern blot analysis.

[00267] Southern blot analysis is performed with genomic DNA obtained from Qiagen DNeasy kit. A total of 2 μg of genomic leaf DNA or 10 μg of genomic callus DNA is subjected to an overnight digestion using BSM I and SWA I restriction enzymes to obtain PTU data .

[00268] After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85 % agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100

microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60°C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

[00269] 8.6 – Postemergence herbicide tolerance in AAD-12 transformed T_0 Corn.

[00270] Four T_0 events were allowed to acclimate in the greenhouse and were grown until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of either Pursuit® (imazethapyr) or 2,4-D Amine 4. Pursuit® was sprayed to demonstrate the function of the selectable marker gene present within the events tested. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. Plants were sprayed with either a lethal dose of imazethapyr (70 g ae/ha) or a rate of 2,4-D DMA salt capable of significant injury to untransformed corn lines (2240 g ae/ha). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

[00271] Several individuals were safened from the herbicides to which the respective genes were to provide resistance. The individual clone '001' from event "001" (a.k.a.,4101(0)-001-001), however, did incur minor injury but recovered by 14 DAT. Three of the four events were moved forward and individuals were crossed with 5XH751 and taken to the next generation. Each herbicide tolerant plant was positive for the presence of the *AAD-12* coding region (PCR assay) or the presence of the *AHAS* gene (Invader assay) for 2,4-D and imazethapyr-tolerant plants, respectively. AAD-12 protein was detected in all 2,4-D tolerant T_0 plants events containing an intact coding region. The copy number of the transgene(s)

(*AHAS*, and by inference *AAD-12*) varied significantly from 1 to 15 copies. Individual T_0 plants were grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T_1 seed.

Table 15. Characterization of T_0 corn plants transformed with *AAD-12*.

Event	Spray Treatment	% Injury (14 DAT)	AAD-12 ELISA (ppm TSP)	AAD12 PCR (Coding Region)	AAD12 PCR (PTU)	AHAS Copy # (Invader)
4101(0)003. 001	2240 g ae/ha 2,4-D	0	146.9	+	+	1
4101(0)003.003	2240 g ae/ha 2,4-D	0	153.5	+	+	1
4101(0)005.001	2240 g ae/ha 2,4-D	0	539.7	+	+	9
4101(0)005.0012	0 g ae/ha 2,4-D	0	562.9	+	+	7
4101(0)001.001	70 g ae/ha imazethapyr	5	170.7	+	+	6
4101(0)002.001	0 g ae/ha imazethapyr	0	105.6	+	-	2
4101(0)002.002	70 g ae/ha imazethapyr	0	105.3	+	-	2
4101(0)003.002	70 g ae/ha imazethapyr	0	0	+	band smaller than expected	15

[00272] 8.7 – Verification of high 2,4-D tolerance in T_1 corn.

[00273] T_1 *AAD-12 (v1)* seed were planted into 3-inch pots containing Metro Mix media and at 2 leaf stage were sprayed with 70 g ae/ha imazethapyr to eliminate nulls. Surviving plants were transplanted to 1-gallon pots containing Metro Mix media and placed in the same growth conditions as before. At V3-V4 stage the plants were sprayed in the track sprayer set to 187 L/ha at either 560 or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to 5XH751 x Hi II control plants. A grading scale of 0-10 (no injury to extreme auxin injury) was developed to distinguish brace root injury. Brace Root grades were taken on 14DAT to show 2,4-D tolerance. 2,4-D causes brace root malformation, and is a consistent indicator of auxinic herbicide injury in corn. Brace root data (as seen in the table below) demonstrates that 2 of the 3 events tested were robustly tolerant to 2240 g ae/ha 2,4-D DMA. Event "pDAB4101(0)001.001" was apparently unstable; however, the other two events were robustly tolerant to 2,4-D and 2,4-D + imazethapyr or 2,-4D + glyphosate (see Table 16).

Table 16. Brace Root injury of *AAD-12 (v1)* transformed T_1 plants and Untransformed control corn plants. A scale of 0-10, 10 being the highest, was used for grading the 2,4-D DMA injury. Results are a visual average of four replications per treatment.

Herbicide	Untransformed Control	*AAD-12 (v1)* pDAB4101(0)003.003	*AAD-12 (v1)*pDAB4101(0)001.001	*AAD-12 (v1)* pDAB4101(0)005.001
	Average Brace Root Injury (0-10 Scale)			
0 g ae/ha 2,4-D DMA	0	0	0	0
2240 g ae/ha 2,4-D DMA	9	1	8	0

[00274] 8.8 – *AAD-12 (v1)* heritability in corn.

[00275] A progeny test was also conducted on seven *AAD-12 (v1)* T_1 families that had been crossed with 5XH751. The seeds were planted in three-inch pots as described above. At the 3 leaf stage all plants were sprayed with 70 g ae/ha imazethapyr in the track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Four out of the six lines tested segregated as a single locus, dominant Mendelian trait (1R:1S) as determined by Chi square analysis. Surviving plants were subsequently sprayed with 2,4-D and all plants were deemed tolerant to 2,4-D (rates ≥ 560 g ae/ha). AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species when reciprocally crossed to a commercial hybrid.

[00276] 8.9 – Stacking of *AAD-12 (v1)* to increase herbicide spectrum

[00277] *AAD-12 (v1)* (pDAB4101) and and elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F_1 seed was collected. The seed from two F_1 lines were planted and treated with 70 g ae/ha imazethapyr at the V2 stage to eliminate nulls. To the surviving plants, reps were separated and either treated with 1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr (to confirm presence of AHAS gene) or 1120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate (to confirm the presence of the Round Up Ready gene) in a track sprayer calibrated to 187 L/ha. Plants were graded 3 and 16 DAT. Spray data showed that *AAD-12 (v1)* can be conventionally stacked with a glyphosate tolerance gene (such as the Roundup CP4-EPSPS gene) or other herbicide tolerance genes to provide an increased spectrum of herbicides that may be applied safely to corn. Likewise imidazolinone + 2,4-D +glyphosate

tolerance was observed in F_1 plants and showed no negative phenotype by the molecular or breeding stack combinations of these multiple transgenes.

Table 17. Data demonstrating increase herbicide tolerance spectrum resulting from an F_1 stack of *AAD-12 (v1)* and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).

Herbicide	**Untransformed Control**	**2P782 (*Roundup Ready* Control)**	***AAD-12 (v1)* pDAB4101(0) 003.R003.AF**	***AAD-12 (v1)* pDAB4101(0) 005.R001.AF**
	Average % Injury 16DAT			
0 g ae/ha 2,4-D DMA	0	0	0	0
1120 g ae/ha 2,4-D DMA	21	19	0	0
1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr	100	100	5	1
01120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate	100	71	2	5

[00278] 8.10- Field tolerance of pDAB4101 transformed corn plants to 2,4-D, triclopyr and fluroxypyr herbicides.

[00279] Field level tolerance trials were conducted on two *AAD-12 (v1)* pDAB4101 events (4101(0)003.R.003.AF and 4101(0)005.R001.AF) and one Roundup Ready (RR) contol hybrid (2P782) at Fowler, Indiana and Wayside, Mississippi. Seeds were planted with cone planter on 40-inch row spacing at Wayside and 30 inch spacing at Fowler. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. The *AAD-12 (v1)* events contained the *AHAS* gene as a selectable marker. The F_2 corn events were segregating so the *AAD-12 (v1)* plants were treated with imazethapyr at 70 g ae/ha to remove the null plants. Herbicide treatments were applied when corn reached the V6 stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after treatment. Brace root injury ratings were taken at 28DAT on a scale of 0-10 with 0-1 being slight brace root fusing, 1-3 being moderate brace root swelling/wandering and root proliferation, 3-5 being moderate brace root fusing, 5-9 severe brace root fusing and malformation and 10 being total inhibition of brace roots.

[00280] *AAD-12 (v1)* event response to 2,4-D, triclopyr, and fluroxypyr at 14 days after treatment are shown in Table 18. Crop injury was most severe at 14 DAT. The RR control

corn (2P782) was severely injured (44% at 14 DAT) by 2,4-D at 4480 g ae/ha, which is 8 times (8X) the fnormal field use rate. The *AAD-12 (v1)* events all demonstrated excellent tolerance to 2,4-D at 14 DAT with 0% injury at the 1, 2 and 4X rates, respectively. The control corn (2P782) was severely injured (31% at 14 DAT) by the 2X rate of triclopyr (840 g ae/ha). *AAD-12 (v1)* events demonstrated tolerance at 2X rates of triclopyr with an average of 3% injury at 14 DAT across the two events. Fluroxypyr at 280 g ae/ha caused 11% visual injury to the wild-type corn at 14 DAT. *AAD-12 (v1)* events demonstrated increased tolerance with an average of 8% injury at 5 DAT.

[00281] Applications of auxinic herbicides to corn in the V6 growth stage can cause malformation of the brace roots. Table 18 shows the severity of the brace root injury caused by 2,4-D, triclopyr, and fluroxypyr. Triclopyr at 840 g ae/ha caused the most severe brace root fusing and malformation resulting in an average brace root injury score of 7 in the 2P782 control-type corn. Both *AAD-12 (v1)* corn events showed no brace root injury from the triclopyr treatment. Brace root injury in 2P782 corn increased with increasing rates of 2,4-D. At 4480 g ae/ha of 2,4-D, the *AAD-12* events showed no brace root injury; whereas, severe brace root fusing and malformation was seen in the 2P782 hybrid. Fluroxypyr caused only moderate brace root swelling and wandering in the wild-type corn with the *AAD-12 (v1)* events showing no brace root injury.

[00282] This data clearly shows that *AAD-12(v1)* conveys high level tolerance in corn to 2,4-D, triclopyr and fluroxypyr at rates far exceeding those commercially used and that cause non-*AAD-12 (v1)* corn severe visual and brace root injury.

Table 18. Visual Injury of AAD-12 events and wild-type corn following foliar applications of 2,4-D, triclopyr and fluroxypyr under field conditions.

		%Visual Injury 14DAT		
Treatment	Rate (g ae/ha)	AAD-12 4101(0)003.R.003.AF	AAD-124101(0)005.001.AF	2P782 control
Untreated	0	0	0	0
2,4-D	1120	0	0	9
2,4-D	2240	0	1	20
2,4-D	4480	0	1	34
Fluroxypyr	280	1	5	11
Triclopyr	840	3	4	31
Dicamba	840	8	8	11

Table 19. Brace root injury ratings for AAD-12 and wild-type corn plants in response to 2,4-D, triclopyr and fluroxypyr under field conditions.

		Brace Root Injury Rating (0-10 scale) 28DAT		
Treatment	Rate (g ae/ha)	AAD-12 event 4101(0)003.R.003.AF	AAD-12 event 4101(0)005.001.AF	Wild-type NK603
Untreated	0	0	0	0
2,4-D	1120	0	0	3
2,4-D	2240	0	0	5
2,4-D	4480	0	0	6
Fluroxypyr	280	0	0	2
Triclopyr	840	0	0	7
Dicamba	840	1	1	1

Example 9. Protein detection from transformed plants via antibody

[00283] 9.1 – Extracting AAD-12 (v1) from plant leaves.

Approximately 50 to 100 mg of leaf tissue was cut into small pieces (or 4 single-hole-punched leaf discs) and put into 2-ml cluster tubes containing 2 stainless steel BB beads (4.5 mm; Daisy Co., cat. #145462-000). Five hundred microliters of plant extraction buffer (PBS containing 0.05 % Tween 20 and 1% Bovine serum albumin) was added to each sample. The tubes were capped and secured in the Geno/Grinder (Model 2000-115, Certiprep, Metuchen, NJ) and shaken for 6 min with setting at 1x of 500 rpm. Tubes were centrifuged at 5000x g for 10 min and supernatant containing the soluble proteins were analyzed for AAD-12 (v1) using Western Blots and ELISA.

[00284] 9.2 – Enzyme linked immuno-sorbent assay (ELISA).

[00285] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-12 antibody (0.5 µg/ml) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 µL standard AAD-12 of known concentrations or plant extracts from different samples were incubated in the wells. For standard curve, purified AAD-12 was diluted 2-fold serially from 52 to 0.813 ng/ml in triplicates. Plant extracts were diluted 5, 10, 20, and 40-fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-12 antibody-HRP conjugate (0.5 ug/ml) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-

Step™ Ultra TMB-ELISA (Pierce, Rockford, IL), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 µL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-12 (v1) in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[00286] For comparison, each sample was normalized with its fresh weight and percent expression was calculated.

[00287] 9.3 – Western blotting analysis.

[00288] Plant extracts or AAD-12 standards (5 and 0.5 µg/ml) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-12 (v1) protein was detected by anti-AAD-12 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham, NJ).

Example 10 – Tobacco Transformation

[00289] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. KY160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB3278, aka pDAS1580, *AAD-12 (v1) + PAT*), grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing

250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30°C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

[00290] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[00291] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-12 (v1)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00292] The DNA samples were diluted to 9 ng/µl and then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the %

signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

[00293] All events were also assayed for the presence of the *AAD-12 (v1)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR *AAD-12* were (SdpacodF: ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:12) and (SdpacodR: CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:13). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably *AAD-12 (v1)* since these genes are physically linked) were regenerated and moved to the greenhouse.

[00294] 10.1 Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T_0 tobacco

[00295] T_0 plants from each of the 19 events were challenged with a wide range of 2,4-D, triclopyr, or fluroxypyr sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluroxypyr was likewise applied at 35, 140, or 560 g ae/ha. Triclopyr was applied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ae/ha (equivalent to 4X field rate). On the whole, *AAD-12 (v1)* events were more sensitive to fluroxypyr, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T_0 plant responses to 560 g ae/ha fluroxypyr. Events were categorized into "low" (>40% injury 14 DAT), "medium" (20-40% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable."

Table 20. Tobacco T0 events transformed with pDAS1580 (*AAD-12 (v1) + PAT*)

# Tube	Plant ID	Copy # *PAT*	PTU PCR *AAD-12*	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance@
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+	*	*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable
24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High

@ Distinguishing herbicide tolerance performance of events required assessment of relative tolerance when treated with 560 g ae/ha fluroxypyr where tolerance was variable across events.

[00296] 10.2 Verification of high 2,4-D tolerance in T_1 tobacco.

[00297] Two to four T_0 individuals surviving high rates of 2,4-D and fluroxypyr were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 7.4), followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (*PAT* gene selection). Survivors were transferred to individual 3-

inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY160 tobacco. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 140 - 2240 g ae/ha 2,4-D dimethylamine salt (DMA), 70-1120 g ae/ha triclopyr or 35-560 g ae/ha fluroxypyr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity.

[00298] No injury was observed at 4X field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line, but the greatest injury was observed with fluroxypyr. The fluroxypyr injury was short-lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT (Table 21). It is important to note that untransformed tobacco is exceedingly sensitive to fluroxypyr. These results indicated commercial level 2,4-D tolerance can be provided by *AAD-12 (v1)*, even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluroxypyr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

Table 21. Assessment of cross tolerance of *AAD-12 (v1)* T_1 tobacco plants' response to various phenoxy and pyridyloxy auxin herbicides.

Herbicide	KY160 – Wildtype	1580(1)-004 (high tolerance in T_0 generation)	1580(1)-018 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
140 g ae/ha 2,4-D DMA	45	0	0
560 g ae/ha 2,4-D DMA	60	0	0
2240 g ae/ha 2,4-D DMA	73	0	0
70 g ae/ha triclopyr	40	0	5
280 g ae/ha triclopyr	65	0	5
1120 g ae/ha triclopyr	80	0	8
35 g ae/ha fluroxypyr	85	0	8
140 g ae/ha fluroxypyr	93	0	10
560 g ae/ha fluroxypyr	100	3	18

[00299] 10.3 *AAD-12 (v1)* heritability in tobacco

[00300] A 100 plant progeny test was also conducted on seven T_1 lines of *AAD-12 (v1)* lines. The seeds were stratified, sown, and transplanted with respect to the procedure above with the exception that null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

10.4 – Field tolerance of pDAS1580 tobacco plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

[00301] Field level tolerance trials were conducted on three *AAD-12 (v1)* lines (events pDAS1580-[1]-018.001, pDAS1580-[1]-004.001 and pDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Mississippi. Tobacco transplants were grown in the greenhouse by planting T_1 seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

[00302] The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after treatment.

[00303] *AAD-12 (v1)* event response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 22. The non-transformed tobacco line was severely injured (63% at 14 DAT) by 2,4-D at 560 g ae/ha which is considered the 1X field application rate. The *AAD-12 (v1)* lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8X rates, respectively. The non-transformed tobacco line was severely injured (53% at 14 DAT) by the 2X rate of triclopyr (840 g ae/ha); whereas, *AAD-*

12 (v1) lines demonstrated tolerance with an average of 5% injury at 14 DAT across the three lines. Fluroxypyr at 280 g ae/ha caused severe injury (99%) to the non-transformed line at 14 DAT. *AAD-12 (v1)* lines demonstrated increased tolerance with an average of 11% injury at 14 DAT.

[00304] These results indicate that *AAD-12 (v1)* transformed event lines displayed a high level of tolerance to 2,4-D, triclopyr and fluroxypyr at multiples of commercial use rates that were lethal or caused severe epinastic malformations to non-transformed tobacco under representative field conditions.

Table 22. *AAD-12 (v1)* tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

Herbicide Treatment		Average % Injury across locations at 14 DAT			
Active Ingredient	Rate	Wild type	pDAS1580-[1]-004.001	pDAS1580-[1]-020.016	pDAS1580-[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxypyr	280 GM AE/HA	99	11	11	12

[00305] 10.5 *AAD-12 (v1)* protection against elevated 2,4-D rates

[00306] Results showing *AAD-12 (v1)* protection against elevated rates of 2,4-D DMA in the greenhouse are shown in Table 23. T_1 *AAD-12 (v1)* plants from an event segregating 3R:1S when selected with 560 g ai/ha Liberty using the same protocol as previously described. T_1 *AAD-1 (v3)* seed was also planted for transformed tobacco controls (*see* PCT/US2005/014737). Untransformed KY160 was served as the sensitive control. Plants were sprayed using a track sprayer set to 187 L/ha at 140, 560, 2240, 8960, and 35840 g ae/ha 2,4-D DMA and rated 3 and 14 DAT.

[00307] *AAD-12 (v1)* and *AAD-1 (v3)* both effectively protected tobacco against 2,4-D injury at doses up to 4X commercial use rates. *AAD-12 (v1),* however, clearly demonstrated a marked advantage over *AAD-1 (v3)* by protecting up to 64X the standard field rates.

Table 23. Results demonstrating protection provided by *AAD-12 (v1)* and *AAD-1 (v3)* against elevated rates of 2,4-D.

	KY160 control	*AAD-1(v3)*	*AAD-12 (v1)*
Treatment	Average % injury 14 DAT		
2240 g ae/ha 2,4-D	95	4	0
8960 g ae/ha 2,4-D	99	9	0
35840 g ae/ha 2,4-D	100	32	4

[00308] 10.6 Stacking of *AAD-12* to increase herbicide spectrum

[00309] Homozygous *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) plants (*see* PCT/US2005/014737 for the latter) were both reciprocally crossed and F_1 seed was collected. The F_1 seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regimine as used for the other testing with one of the following treatments: 70, 140, 280 g ae/ha fluroxypyr (selective for the *AAD-12 (v1)* gene); 280, 560, 1120 g ae/ha R-dichloroprop (selective for the *AAD-1 (v3)* gene); or 560, 1120, 2240 g ae/ha 2,4-D DMA (to confirm 2,4-D tolerance). Homozygous T_2 plants of each gene were also planted for use as controls. Plants were graded at 3 and 14 DAT. Spray results are shown in Table 24.

[00310] The results confirm that *AAD-12 (v1)* can be successfully stacked with *AAD-1 (v3)*, thus increasing the spectrum herbicides that may be applied to the crop of interest (phenoxyactetic acids + phenoxypropionic acids vs penoxyacetic acids + pyridyloxyacetic acids for AAD-1 and AAD-12, respectively). The complementary nature of herbicide cross resistance patterns allows convenient use of these two genes as complementary and stackable field-selectable markers. In crops where tolerance with a single gene may be marginal, one skilled in the art recognizes that one can increase tolerance by stackinga second tolerance gene for the same herbicide. Such can be done using htesame gene with the same or different promoters; however, as observed here, stacking and tracking two completmentary traits can be facilitated by the distinguishing cross protection to phenoxypropionic acids [from *AAD-1 (v3)*] or pyidyloxyacetic acids [*AAD-12 (v1)*]

Table 24. Comparison of auxinic herbicide cross tolerance of *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) T_2 plants compared to AAD-12 x AAD-1 F1 cross and to wildtype.

Treatment	KY160 wildtype control	*AAD-12 (v1)* (pDAS1580)	*AAD-1(v3)* (pDAB721)	*AAD-12 (v1)* x *AAD (v3)* F_1
	Average % injury 14 DAT			
560 g ae/ha 2,4-D	63	0	0	0
1120 g ae/ha 2,4-D	80	0	4	0
2240 g ae/ha 2,4-D	90	0	9	0
280 g ae/ha R-dichlorprop	25	15	0	0
560 g ae/ha R-dichlorprop	60	50	0	0
1120 g ae/ha R-dichlorprop	80	70	3	0
70 g ae/ha fluroxypyr	40	0	40	0
140 g ae/ha fluroxypyr	65	0	60	0
280 g ae/ha fluroxypyr	75	3	75	3

Example 11 - Soybean Transformation

[00311] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00312] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean,

zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-12 (v1)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-12 (v1)*.

[00313] 11.1 Gateway cloning of binary constructs

[00314] The *AAD-12 (v1)* coding sequence was cloned into five different Gateway Donor vectors containing different plant promoters. The resulting *AAD-12 (v1)* plant expression cassettes were subsequently cloned into a Gateway Destination Binary vector via the LR Clonase reaction (Invitrogen Corporation, Carlsbad Ca, Cat # 11791-019).

[00315] An NcoI – SacI fragment containing the *AAD-12 (v1)* coding sequence was digested from DASPICO12 and ligated into corresponding NcoI – SacI restriction sites within the following Gateway Donor vectors: pDAB3912 (attL1 // CsVMV promoter // AtuORF23 3'UTR // attL2); pDAB3916 (attL1 // AtUbi10 promoter // AtuORF23 3'UTR // attL2); pDAB4458 (attL1 // AtUbi3 promoter // AtuORF23 3'UTR // attL2); pDAB4459 (attL1 // ZmUbi1 promoter // AtuORF23 3'UTR // attL2); and pDAB4460 (attL1 // AtAct2 promoter // AtuORF23 3'UTR // attL2). The resulting constructs containing the following plant expression cassettes were designated: pDAB4463 (attL1 // CsVMV promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2); pDAB4467 (attL1 // AtUbi10 promoter // AAD-12 (v1) // AtuORF23 3'UTR // attL2); pDAB4471 (attL1 // AtUbi3 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2); pDAB4475 (attL1 // ZmUbi1 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2); and pDAB4479 (attL1 // AtAct2 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2). These constructs were confirmed via restriction enzyme digestion and sequencing.

[00316] The plant expression cassettes were recombined into the Gateway Destination Binary vector pDAB4484 (RB7 MARv3 // attR1 – ccdB – chloramphenicol resistance – attR2 //

CsVMV promoter // PATv6 // AtuORF1 3'UTR) via the Gateway LR Clonase reaction. Gateway Technology uses lambda phage-based site-specific recombination instead of restriction endonuclease and ligase to insert a gene of interest into an expression vector. Invitrogen Corporation, Gateway Technology: A Universal Technology to Clone DNA Sequences for Functional Analysis and Expression in multiple Systems, Technical Manual, Catalog #'s 12535-019 and 12535-027, Gateway Technology Version E, 9/22/2003, #25-022. The DNA recombination sequences (attL, and attR,) and the LR Clonase enzyme mixture allows any DNA fragment flanked by a recombination site to be transferred into any vector containing a corresponding site. The attL1 site of the donor vector corresponds with attR1 of the binary vector. Likewise, the attL2 site of the donor vector corresponds with attR2 of the binary vector. Using the Gateway Technology the plant expression cassette (from the donor vector) which is flanked by the attL sites can be recombined into the attR sites of the binary vector. The resulting constructs containing the following plant expression cassettes were labeled as: pDAB4464 (RB7 MARv3 // CsVMV promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR); pDAB4468 (RB7 MARv3 // AtUbi10 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR); pDAB4472 (RB7 MARv3 // AtUbi3 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter// *PATv6* // AtuORF1 3'UTR); pDAB4476 (RB7 MARv3 // ZmUbi1 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR); and pDAB4480 (RB7 MARv3 // AtAct2 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR) (see Table 8). These constructs were confirmed via restriction enzyme digestion and sequencing.

[00317] 11.2 Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00318] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin

followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00319] 11.2.1 – Plant transformation production of *AAD-12 (v1)* tolerant phenotypes. Seed derived explants of "Maverick"and the *Agrobacterium* mediated cot-node transformation protocol was used to producw *AAD-12 (v1)* transgenic plants.

[00320] 11.2.2– *Agrobacterium* preparation and inoculation

[00321] *Agrobacterium* strain EHA101 (Hood et al. 1986), carrying each of five binary pDAB vectors (Table 8) was used to initiate transformation. Each binary vector contains the *AAD-12 (v1)* gene and a plant-selectable gene (*PAT*) cassette within the T-DNA region. Each gene is driven by the promoters listed in Table 8 and these plasmids were mobilized into the EHA101 strain of *Agrobacterium* by electroporation. The selected colonies were then analyzed for the integration of genes before the Agrobacterium treatment of the soybean explants. Maverick seeds were used in all transformation experiments and the seeds were obtained from University of Missouri, Columbia, MO.

[00322] Agrobacterium-mediated transformation of soybean (*Glycine max*) using the *PAT* gene as a selectable marker coupled with the herbicide glufosinate as a selective agent was carried out followed a modified procedure of Zeng et al. (2004). The seeds were germinated on B5 basal medium (Gamborg et al. 1968) solidified with 3 g/L Phytagel (Sigma-Aldrich, St. Louis, Mo.); added l-cysteine to the co-cultivation medium at 400 mg/L and co-cultivation

lasted 5 days (Olhoft and Somers 2001); shoot initiation, shoot elongation, and rooting media were supplemented with 50 mg/L cefotaxime, 50 mg/L timentin, 50 mg/L vancomycin, and solidified with 3 g/L Phytagel. Selected shoots were then transferred to the rooting medium. The optimal selection scheme was the use of glufosinate at 8 mg/L across the first and second shoot initiation stages in the medium and 3–4 mg/L during shoot elongation in the medium.

[00323] Prior to transferring elongated shoots (3–5 cm) to rooting medium, the excised end of the internodes were dipped in 1 mg/L indole 3-butyric acid for 1–3 min to promote rooting (Khan et al. 1994). The shoots struck roots in 25×100 mm glass culture tubes containing rooting medium and then they were transferred to soil mix for acclimatization of plantlets in Metro-mix 200 (Hummert International, Earth City, Mo.) in open Magenta boxes in Convirons. Glufosinate, the active ingredient of Liberty herbicide (Bayer Crop Science), was used for selection during shoot initiation and elongation. The rooted plantlets were acclimated in open Magenta boxes for several weeks before they were screened and transferred to the greenhouse for further acclimation and establishment.

[00324] 11.2.3 - Assay of putatively transformed plantlets, and analyses established T_0 plants in the greenhouse.

[00325] The terminal leaflets of selected leaves of these plantlets were leaf painted with 50 mg/L of glufosinate twice with a week interval to observe the results to screen for putative transformants. The screened plantlets were then transferred to the greenhouse and after acclimation the leaves were painted with glufosinate again to confirm the tolerance status of these plantlets in the GH and deemed to be putative transformants.

[00326] Plants that are transferred to the greenhouse can be assayed for the presence of an active *PAT* gene further with a non-destructive manner by painting a section of leaf of the T_0 primary transformant, or progeny thereof, with a glufosinate solution [0.05-2% v/v Liberty Herbicide, preferably 0.25-1.0% (v/v),=500-2000 ppm glufosinate, Bayer Crop Science]. Depending on the concentration used, assessment for glufosinate injury can be made 1-7 days after treatment. Plants can also be tested for 2,4-D tolerance in a non-destructive manner by selective application of a 2,4-D solution in water (0.25-1% v/v commercial 2,4-D dimethylamine salt formulation, preferably 0.5% v/v = 2280 ppm 2,4-D ae) to the terminal leaflet of the newly expanding trifoliolate one or two, preferably two, nodes below the youngest emerging trifolioate. This assay allows assessment of 2,4-D sensitive plants 6 hours to several days after application by assessment of leaf flipping or rotation >90 degrees from

the plane of the adjacent leaflets. Plants tolerant to 2,4-D will not respond to 2,4-D. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-12 (v1)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically comprise one or two selective rates in the range of 100-1120 g ae/ha using a track sprayer as previously described. T_1 plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance (in plants transformed with constructs that contain EPSPS) or another glyphosate tolerance gene can be assessed in the T_1 generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha glyphosate. Analysis of protein expression will occur as described in below. Individual T_0 plants were assessed for the presence of the coding region of the gene of interest (*AAD-12 (v1)* or *PAT v6*) and copy number. Determination of the inheritance of *AAD-12 (v1)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance as described in previous examples.

[00327] A subset of the initial transformants were assessed in the T_0 generation according to the methods above. Any plant confirmed as having the *AAD-12 (v1)* coding region, regardless of the promoter driving the gene did not respond to the 2,4-D leaf painting whereas wildtype Maverick soybeans did (Table Sec 11.2.3). *PAT*-only transformed plants responded the same at wildtype plants to leaf paint applications of 2,4-D

[00328] 2,4-D was applied to a subset of the plants that were of similar size to the wildtype control plants with either 560 or 1120 g ae 2,4-D. All *AAD-12 (v1)*-containing plants were clearly resistant to the herbicide application versus the wildtype Maverick soybeans. A slight level of injury (2 DAT) was observed for two *AAD-12 (v1)* plants, however, injury was temporary and no injury was observed 7 DAT. Wildtpe control plants were severely injured 7-14 DAT at 560 g ae/ha 2,4-D and killed at 1120 g ae/ha. These data are consistent with the fact that *AAD-12 (v1)* can impart high tolerance (>2X field rates) to a sensitive crop like soybeans. The screened plants were then sampled for molecular and biochemical analyses for the confirmation of the *AAD12 (v1)* genes integration, copy number, and their gene expression levels as described below and reported in Table 25.

Table 25. T_0 soybean response to 2,4-D leaf paint and 2,4-D spray application.

Construct (pDAB#)	Gene	Promoter	Event	NODE LEAF PAINTED	Leaf flip assay 2,4-D @ (18 HAT) Node N-1	Node N-2	Spray POST over the top with 2,4-D (g ae/ha)	Stage at appl (# nodes)	ELISA] (ng/mL)	Southern Copy number	PCR coding region	% injury 2 DAT	% injury 7 DAT	% injury 14 DAT
4464	AAD-12	CsVMV	D-1-14	N-1	0		0	>10	5246.83	2	+	X	X	0
4464	AAD-12	CsVMV	D-2-9	N-2		0	0	>10	204.27	1	+	X	X	0
4468	AAD-12	AtUbi10	D-3-7	N-2		0	0	>10	4.65	1	+	0	0	0
4468	AAD-12	AtUbi10	D-4-11B	N-2		0	0	8	1452.84	2	+	0	0	0
4468	AAD-12	AtUbi10	D-4-16	N-2		0	0	>10	653.21	2	+	X	X	0
4480	AAD-12	AtAct2	D-9-1	N-2		0	0	>10	248.33	3 or 4	+	X	X	0
4464	AAD-12	CsVMV	D-2-14	N-2		0	560	7	4917.43	2	+	0	0	0
4468	AAD-12	AtUbi10	D-3-5	N-2		0	560	8	365.75	1	+	0	0	0
4468	AAD-12	AtUbi10	D-3-6	N-1	0		560	5	714.79	3	+	0	0	0
4472	AAD-12	AtUbi3	D-5-2	N-1	0		560	6	0.58	1	+	5	0	0
4468	AAD-12	AtUbi10	D-3-9	N-2		0	1120	6	2657.26	3	+	0	0	0
4468	AAD-12	AtUbi10	D-4-17	N-2		0	1120	7	286.14	5	+	5	0	0
4499	PAT	CsVMV	D-2-3	N-2		1	0	>10	2.36	5	+	X	X	0
Maverick	WT		WT-10	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-2	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-3	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-4	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-5	NT			560	4	ND	ND	ND	50	60	60
Maverick	WT		WT-6	NT			560	4	ND	ND	ND	70	90	80
Maverick	WT		WT-7	NT			560	4	ND	ND	ND	70	80	80
Maverick	WT		WT-10	NT			1120	4	ND	ND	ND	70	90	100
Maverick	WT		WT-8	NT			1120	4	ND	ND	ND	70	95	100
Maverick	WT		WT-9	NT			1120	4	ND	ND	ND	70	95	100
					1=Flip				ND=Not determined					
					0=No Flip									

[00329] 11.2.4– Molecular Analyses: Soybean

[00330] 11.2.4.1– Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μL.

[00331] 11.2.4.2- Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-12 (v1)* PTU are (Forward – ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:8) and (Reverse – CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR AAD-12 (v1) are (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:10) and (Reverse – CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00332] 11.2.4.3- Southern blot analysis. Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 10 μg of genomic DNA is subjected to an overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85 % agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene

UV stratalinker 1800) at 1200 X100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

[00333] 11.2.5 – Biochemical Analyses: Soybean

[00334] 11.2.5.1 – Tissue Sampling and Extracting AAD-12 (v1) protein from soybean leaves. Approximately 50 to 100 mg of leaf tissue was sampled from the N-2 leaves that were 2,4-D leaf painted, but after 1 DAT. The terminal N-2 leaflet was removed and either cut into small pieces or 2-single-hole- punched leaf discs (~0.5 cm in diameter) and were frozen on dry ice instantly. Further protein analysis (ELISA and Western analysis) was completed according to methods described in Example 9.

[00335] 11.2.6 – T_1 Progeny evaluation. T_0 plants will be allowed to self fertilize to derive T_1 families. Progeny testing (segregation analysis) will be assayed using glufosinate at 560 g ai/ha as the selection agent applied at the V1-V2 growth stage. Surving plants will be further assayed for 2,4-D tolerance at one or more growth stages from V2-V6. Seed will be produced through self fertilization to allow broader herbicide testing on the transgenic soybean.

[00336] *AAD-12 (v1)* transgenic Maverick soybean plants have been generated thorugh *Agrobacterium*- mediated cot-node transformation system. The T_0 plants obtained tolerated up to 2X levels of 2,4-D field applications and developed fertile seeds. The frequency of fertile transgenic soybean plants was up to 5.9%. The integration of the *AAD1-12 (v1)* gene into the soybean genome was confirmed by Southern blot analysis. This analysis indicated that most of the transgenic plants contained a low copy number. The plants screened with

AAD-12 (v1) antibodies showed positive for ELISA and the appropriate band in Western analysis.

[00337] 11.3 Transformation Method 2: Aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00338] Culture of embryogenic soybean callus tissue and subsequent beaming can be accomplished as described in U.S. Patent No. 6,809,232 (Held *et al.*) to create transformants using one or more constructs in Table 8.

[00339] 11.4 Transformation Method 3. Biolistic bombardment of Soybean

[00340] This can be accomplished using mature seed derived embryonic axes meristem (McCabe et al. (1988)). Following established methods of biolistic bombardment, one can expect recovery of transformed soyben plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

[00341] 11.5 Transformation Method 4. Whiskers mediated transformation.

[00342] Whisker preparation and whisker transformation can anticipated according to methods described previously by Terakawa et al. (2005)). Following established methods of biolistic bombardment, one can expect recovery of transformed soyben plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

[00343] Maverick seeds were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 min. and then rinsed three times in sterile distilled water. The seeds were soaked in distilled water for 18–20 h. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were positioned in the bombardment medium [BM: MS (Murashige and Skoog 1962) basal salts medium, 3% sucrose and 0.8% phytagel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium.

[00344] 11.6 Transformation Method 5. Particle bombardment-mediated transformation for embryogenic callus tissue can be optimized for according to previous methods (Khalafalla et al., 2005; El-Shemy et al., 2004, 2006). Regenrated plants can also be assessed according to Example 11.2.

Example 12– *AAD-12 (v1)* in Cotton

[00345] 12.1 – Cotton transformation protocol.

[00346] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (Table 26) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00347] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 26) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (Table 26) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 26). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00348] *Agrobacterium* preparation. Inoculate 35ml of Y media (Table 26) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (Table 26) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per ml with a total volume of 40 ml.

[00349] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison, MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.01 and 0.05mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for to the AAD-12 enzyme, however dichlorprop is more active on cotton than 2,4-D. In a separate comparison, segments which were plated on MG media containing no growth regulator compared to standard MG media, showed reduced callusing, but there still is callus growth. Callus is then transferred to CG-media (Table 26), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 26) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed,

and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color. Cotton can take time to regenerate and form embryos, one of the ways to speed up this process is to stress the tissue. Dessication is a common way to accomplish this, via changes in the microenvironment of the tissue and plate, by using less culture media and/or adopting various modes of plate enclosure (taping versus parafilm).

[00350] Larger, well-developed embryos are isolated and transferred to DK media (Table 26) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

Table 26. Media for Cotton Transformation

Ingredients in 1 liter	**G**	**M liquid**	**M**	**MG**	**CG**	**D**	**DK**	**Y**
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
Agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA				2 ml	0.4 ml			
Carbenicillin (250 mg/ml)				0.5 ml	0.3 ml			10 grams
GLA (10mg/ml)								10 grams
Peptone								5 grams
Yeast Extract								
NaCl								

[00351] 12.2 – Cell transformation.

[00352] Several experiments were initiated in which cotyledon segments were treated with *Agrobacterium* containing pDAB724. Over 2000 of the resulting segments were treated using various auxin options for the proliferation of pDAB724 cotton callus, either: 0.1 or 0.5mg/L R-dichlorprop, standard 2,4-D concentration and no auxin treatment. The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage; then callus lines that are embryogenic will be sent for Western analysis, essentially as described in section 11.2.3. Embryogenic cotton callus was stressed using dessication techniques to improve the quality and quantity of the tissue recovered.

[00353] Almost 200 callus events have been screened for intact PTU and expression using Western analysis for the *AAD-12 (v1)* gene. Below is a subset of the data for some of the cotton callus that has been tested.

Construct	Line Number	*AAD-12* PTU	*AAD-12* Invader	AAD-12 ng/ml
pDAB724	1	+	+	79.89
pDAB724	2	+	+	17.34
pDAB724	3	+	+	544.80
pDAB724	4	+	+	32.63
pDAB724	5	+	+	82.77
pDAB724	83	+	+	795.50
pDAB724	84	+	+	613.35
pDAB724	85	+	+	1077.75
pDAB724	86	+	+	437.74
pDAB724	87	+	+	286.51
pDAB724	88	+	+	517.59
pDAB724	89	+	+	1250.70

[00354] 12.3 – Plant regeneration.

[00355] *AAD-12 (v1)* cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the *AAD-12 (v1)* gene provides resistance to 2,4-D in cotton, both the *AAD-12 (v1)* cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187 L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T_1 seed or outcrossed with an elite cotton line to produce F_1 seed. The subsequent seed produced will be planted and evaluated for herbicide

resistance as previously described. AAD-12 (v1) events can be combined with other desired HT or IR trants as described in experimens 18, 19, 22, and 23.

Example 13 – *Agrobacterium* Transformation of Other Crops

[00356] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g.,* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.*, 1997.

[00357] The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *AAD-12 (v1)*, for example, into these and other plants, including but not limited to Maize (*Zea mays*), Wheat (*Triticum* spp.), Rice (*Oryza* spp. and *Zizania* spp.), Barley (*Hordeum* spp.), Cotton (*Abroma augusta* and *Gossypium* spp.), Soybean (*Glycine max*), Sugar and table beets (*Beta spp.*), Sugar cane (*Arenga pinnata*), Tomato (*Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato (*Solanum tubersoum)*, Sweet potato (*Ipomoea betatas)*, Rye (*Secale* spp.), Peppers (*Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Lactuca sativa*, *perennis*, and *pulchella*), Cabbage (*Brassica spp*), Celery (*Apium graveolens*), Eggplant (*Solanum melongena*), Peanut (*Arachis* hypogea), Sorghum (all *Sorghum* species), Alfalfa (*Medicago sativua*), Carrot (*Daucus carota*), Beans (*Phaseolus* spp. and other genera), Oats (*Avena sativa* and *strigosa*), Peas (*Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Helianthus annuus*), Squash (*Cucurbita* spp.), Cucumber (*Cucumis sativa*), Tobacco (*Nicotiana* spp.), *Arabidopsis* (*Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, *Poa, Cynadon,* and other genera), Clover (*Tifolium*), Vetch (*Vicia*). Such plants, with *AAD-12 (v1)* genes, for example, are included in the subject invention.

[00358] *AAD-12 (v1)* has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluroxypyr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited

to: Alder (*Alnus spp.*), ash (*Fraxinus spp.*), aspen and poplar species (*Populus spp.*), beech (*Fagus spp.*), birch (*Betula spp.*), cherry (*Prunus spp.*), eucalyptus (*Eucalyptus spp.*), hickory (*Carya spp.*), maple (*Acer spp.*), oak (*Quercus spp*), and pine (*Pinus spp*). Use of auxin resistance for the selective weed control in ornamental and fruit-bearing species is also within the scope of this invention. Examples could include, but not be limited to, rose *(Rosa spp.),* burning bush (*Euonymus spp.*), petunia (*Petunia spp)*, begonia (*Begonia spp.*), rhododendron (*Rhododendron spp),* crabapple or apple (*Malus spp.*), pear (*Pyrus spp.*), peach (*Prunus spp*), and marigolds (*Tagetes spp.*).

Example 14 – Further Evidence of Surprising Results: *AAD-12 vs. AAD-2*

[00359] 14.1 – *AAD-2 (v1)* initial cloning.

[00360] Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00361] The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC

TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

[00362] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H_2O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00363] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO:6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:7) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[00364] 14.2 – Completion of *AAD-2 (v1)* binary vector.

[00365] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. *See* PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:14) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:15) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing

reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI – NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

[00366] 14.3 – Evaluation of transformed *Arabidopsis*.

[00367] Freshly harvested T_1 seed transformed with a plant optimized *AAD-12 (v1)* or native *AAD-2 (v1)* gene were planted and selected for resistance to glufosinate as previously described Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

[00368] *AAD-12 (v1)* and *AAD -2 (v1)* did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Surprisingly, *AAD-2 (v1)*and *AAD-2 (v2)* transformants were far less resistant to 2,4-D than the *AAD-12 (v1)* gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (*see* PCT/US2005/014737). Conversely, *AAD-12 (v1)* had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D (Table 11). Tolerance improved slightly for plant-optimized *AAD-2 (v2)* versus the native gene; however, comparison of both *AAD-12* and *AAD-2* plant optimized genes indicates a significant advantage for *AAD-12 (v1) in planta.*

[00369] These results are unexpected given that the *in vitro* comparison of *AAD-2 (v1)* (*see* PCT/US2005/014737) and *AAD-12 (v2)* indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual T_1 plants to varying levels; however, little protection

from 2,4-D injury is afforded by this expressed protein. No substantial difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes (*see* PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of *AAD-12 (v1) in planta*, and resulting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides, unexpected.

Example 15 – Preplant Burndown Applications

[00370] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

[00371] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (*see* federal labels, most are available through

CPR, 2005 or online at cdms.net/manuf/manuf.asp). With *AAD-12 (v1)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2005) and uses described in Agriliance Crop Protection Guide (2005), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. Likewise, the unique attributes of AAD-12 allowing degradation of triclopyr and fluroxypyr increase utility by allowing substitution or tank mixesof 70-1120 or 35-560 g ae/ha of triclopyr and fluroxypyr, respectively, to increase spectram and/or increase the abilty to control perennial or viney weed species.

Example 16 – In-Crop Use of Phenoxy Auxins Herbicides in Soybeans, Cotton, and Other Dicot Crops Transformed Only with *AAD-12 (v1)*

[00372] *AAD-12 (v1)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D and MCPA) and pyridyloxy auxins (triclopyr and fluroxypyr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00373] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluroxypyr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

[00374] A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetmide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

[00375] Further benefits could include tolerance to 2,4-D, triclopyr or fluroxypyr required before planting following aryloxyacetic acid auxin herbicide application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D, triclopyr or fluroxypyr. Dicamba (and many other herbicides) can still be used for the subsequent control of *AAD-12 (v1)*-transformed dicot crop volunteers.

[00376] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by *AAD-12 (v1)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 17 - In-Crop Use of Phenoxy Auxin and Pyridyloxy Auxin Herbicides in *AAD-12 (v1)* Only Transformed Corn, Rice, and Other Monocot Species

[00377] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-12 (v1)* would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D,.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. *AAD-12 (v1)*-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00378] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, or fluroxypyr. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-12 (v1)* HTC combination strategy, whether one rotates crops species or not.

[00379] A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetmide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

[00380] The increased tolerance of corn, rice, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage

restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 18 – *AAD-12 (v1)* Stacked With Glyphosate Tolerance Trait in Any Crop

[00381] The vast majority of cotton, canola, corn, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-12 (v1)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed,

or as a premix with glyphosate to provide effective control. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-12 (v1)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Tricopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimin. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D, triclopyr, or fluroxypyr.

[00382] One skilled in the art of weed control will recognize that use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 19 - *AAD-12 (v1)* Stacked with Glufosinate Tolerance Trait in Any Crop

[00383] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and

broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2005).

[00384] By stacking *AAD-12 (v1)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis Apocynum cannabinum, and Conyza candensis*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species. Triclopyr and fluroxypyr would be acceptable components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) A multiple combination of glufosinate (200-500 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00385] One skilled in the art of weed control will recognize that use of one or more commercial aryloxyacetic auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - *AAD-12 (v1)* Stacked with *AHAS* Trait in Any Crop

[00386] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2005). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2005). By stacking *AAD-12 (v1)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monoct crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and these auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2005) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp.* can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. See rates above for triclopyr or fluroxypyr.

b) A mulitple combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00387] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by *AAD-12 (v1)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-12 (v1)* in Rice

[00388] 21.1 – Media description.

[00389] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/L Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

[00390] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/L glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/L sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/L hygromycin B for 3–4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/L 6-benzylaminopurine (BAP), 1 mg/L α-naphthaleneacetic acid (NAA), 5 mg/L abscisic acid (ABA) and 50 mg/L hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/L BAP, 0.5 mg/L NAA, and 50 mg/L hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/L hygromycin B (1/2MSH50).

[00391] 21.2 – Tissue culture development.

[00392] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that

the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week. prior to storage.

[00393] 21.3 – Microprojectile bombardment.

[00394] All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(*Hpt*-containing vector) to pDAB4101 (*AAD-12 (v1)* + *AHAS*), 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[00395] 21.4 – Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T_0 Rice

[00396] Rice plantlets at the 3-5 leaf stage were sprayed with a lethal dose of 0.16% (v/v) solution of Pursuit (to confirm the presence of the *AHAS* gene) containing 1% Sunit II (v/v) and 1.25% UAN (v/v) using a track sprayer calibrated to 187 L/ha. Rating for sensitivity or resistance was performed at 36 days after treatment (DAT). Ten of the 33 events sent to the greenhouse were robustly tolerant to the Pursuit; others suffered varying levels of herbicide injury. Plants were sampled (according to section 21.7 below) and molecular characterization

was performed as previously described in Example 8 that identified seven of these 10 events as containing both the *AAD-12 (v1)* PTU and the entire *AHAS* coding region.

[00397] 21.5 – Heritability of AAD-12 (v1) in T_1 rice

[00398] A 100-plant progeny test was conducted on five T_1 lines of *AAD-12 (v1)* lines that contained both the *AAD-12 (v1)* PTU and *AHAS* coding region. The seeds were planted with respect to the procedure above and sprayed with 140 g ae/ha imazethapyr using a track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Two out of the five lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 coseregated with the *AHAS* selectable marker as determined by 2,4-D tolerance testing below.

[00399] 21.6 – Verification of high 2,4-D tolerance in T_1 Rice.

[00400] The following T_1 *AAD-12 (v1)* single segregating locus lines were planted into 3-inch pots containing Metro Mix media: pDAB4101(20)003 and pDAB4101(27)002. At 2-3 leaf stage were sprayed with 140 g ae/ha imazethapyr. Nulls were eliminated and individuals were sprayed at V3-V4 stage in the track sprayer set to 187 L/ha at 1120, 2240 or 4480 g ae/ha 2,4-D DMA (2X, 4X, and 8X typical commercial use rates, respectively). Plants were graded at 7 and 14 DAT and compared to untransformed commercial rice cultivar, 'Lamont,' as negative control plants.

[00401] Injury data (Table 27) shows that the *AAD-12 (v1)*-transformed lines are more tolerant to high rates of 2,4-D DMA than the untransformed controls. The line pDAB4101(20)003 was more tolerant to high levels of 2,4-D than the line pDAB4101(27)002. The data also demonstrates that tolerance of 2,4-D is stable for at least two generations.

Table 27. T_1 *AAD-12 (v1)* and untransformed control respone to varying levels of 2,4-D DMA.

Herbicide	Lemont Untransformed Control	pDAB4101(20)003	pDAB4101(27)002
	Average % Injury 14 DAT		
1120 g ae/ha 2,4-D DMA	20	10	10
2240 g ae/ha 2,4-D DMA	35	15	30
4480 g ae/ha 2,4-D DMA	50	23	40

[00402] 21.7 – Tissue harvesting, DNA isolation and quantification.

[00403] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

[00404] 21.8 – AAD-12 (v1) expression.

[00405] Sample preparation and analysis conditions were as described previously. All 33 T_0 transgenic rice lines and 1 non-transgenic control were analyzed for AAD-12 expression using ELISA blot. AAD-12 was detected in the clones of 20 lines, but not in line Taipai 309 control plant. Twelve of the 20 lines that had some of the clones tolerant to imazethapyr were expressing AAD-12 protein, were AAD-12 PCR PTU positive, and AHAS coding region positive. Expression levels ranged from 2.3 to 1092.4 ppm of total soluble protein.

[00406] 21.9– Field tolerance of pDAB4101 rice plants to 2,4-D and triclopyr herbicides.

[00407] A field level tolerance trial was conducted with *AAD-12 (v1)* event pDAB4101[20] and one wild-type rice (Clearfield 131) at Wayside, Mississippi (a non-transgenic imidazolinone-resistant variety). The experimental design was a randomized complete block design with a single replication. Herbicide treatments were 2X rates of 2,4-D (dimethylamine salt) at 2240 g ae/ha and triclopyr at 560 g ae/ha plus an untreated control. Within each herbicide treatment, two rows of T_1 generation pDAB4101[20] and two rows of Clearfield rice were planted using a small plot drill with 8-inch row spacing. The pDAB4101[20] rice contained the *AHAS* gene as a selectable marker for the *AAD-12(v1)* gene. Imazethapyr was applied at the one leaf stage as selection agent to remove any *AAD-12 (v1)* null plants from the plots. Herbicide treatments were applied when the rice reached the 2 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual ratings of injury were taken at 7, 14 and 21 days after application.

[00408] *AAD-12 (v1)* event response to 2,4-D and triclopyr are shown in Table 28. The non-transformed rice line (Clearfield) was severely injured (30% at 7DAT and 35% at 15DAT) by 2,4-D at 2240 g ae/ha which is considered the 4X commercial use rate. The *AAD-12 (v1)*

event demonstrated excellent tolerance to 2,4-D with no injury observed at 7 or 15DAT. The non-transformed rice was significantly injured (15% at 7DAT and 25% at 15DAT) by the 2X rate of triclopyr (560 g ae/ha). The *AAD-12 (v1)* event demonstrated excellence tolerance to the 2X rates of triclopyr with no injury observed at either 7 or 15DAT.

[00409] These results indicate that the *AAD-12 (v1)* transformed rice displayed a high level of resistance to 2,4-D and triclopyr at rates that caused severe visual injury to the Clearfield rice. It also demonstrates the ability to stack multiple herbicide tolerance genes with AAD-12 I multiple species to provide resistance to a wider spectrum of effective chemistries

Table 28. AAD-12 T_1 generation rice plants response to 2,4-D and triclopyr under field conditions.

Herbicide Treatment		% Visual Injury			
		7DAT		15DAT	
Active Ingredient	Rate	AAD-12 event pDAB4101[20]	Wild-type Clearfield	AAD-12 event pDAB4101[20]	Wild-type Clearfield
2,4-D	2240 GM AE/HA	0	15	0	35
Triclopyr	840 GM AE/HA	0	30	0	25
Untreated		0	0	0	0

Example 22 – *AAD-12 (v1)* in Canola

[00410] 22.1 – Canola transformation.

[00411] The *AAD-12 (v1)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained *AAD-12 (v1)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi10 promoter (*see* section 2.4).

[00412] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00413] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/L kinetin and 1 mg/L 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00414] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/L Carbenicillin and 300 mg/L Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/L Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00415] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 1 mg/L Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 3 mg/L Herbiace, Carbenicillin and Timentin) for another 2-3 weeks.

[00416] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/L MES, 5 or 10 mg/L Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/L Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00417] 22.2 – Molecular analysis: canola materials and methods

[00418] 22.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green

(Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ul.

[00419] 22.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-12 (v1)* were (SEQ ID NO:10) (forward) and (SEQ ID NO:11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-12 (v1)* events tested positive. Three negative control samples tested negative.

[00420] 22.2.3 – ELISA.

[00421] Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 29.

Table 29. Expression of *AAD-12 (v1)* in Canola plants

Sample #	[TSP] (µg/ml)	[AAD-12] (ng/ml)	Expression (ppm TSP) (ELISA)	Western
31	5614.96	1692.12	301.36	++++
33	4988.26	2121.52	425.30	++++
38	5372.25	3879.09	722.06	++++
39	2812.77	41.36	14.71	+
40	3691.48	468.74	126.98	+++
Control 1	2736.24	0.00	0.00	-
Control 2	2176.06	0.00	0.00	-
Control 3	3403.26	0.00	0.00	-

[00422] 22.4 – Postemergence herbicide tolerance in *AAD-12(v1)* transformed T_0 Canola.

[00423] Forty-five T_0 events from the transformed with the construct pDAB3759, were sent to the greenhouse over a period of time and were allowed to acclimate in the greenhouse. The plants were grown until 2-4 new, normal looking leaves had emerged (i.e., plants had

transitioned from tissue culture to greenhouse growing conditions). Plants were then treated with a lethal dose of the commercial formulations of 2,4-D Amine 4 at a rate of 560 g ae/ha. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. A lethal dose is defined as the rate that causes >95% injury to the untransformed controls.

[00424] Twenty-four of the events were tolerant to the 2,4-D DMA herbicide application. Some events did incur minor injury but recovered by 14 DAT. Events were progressed to the T_1 (and T_2 generation) by selfpollination under controlled, bagged, conditions.

[00425] 22.5 – *AAD-12 (v1)* heritability in Canola.

[00426] A 100 plant progeny test was also conducted on 11 T_1 lines of *AAD-12 (v1)*. The seeds were sown and transplanted to 3-inch pots filled with Metro Mix media. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Seven out of the 11 lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi-square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species and can be stacked with one or more additional herbicide resistance genes.

[00427] 22.6 – Verification of high 2,4-D tolerance in T_1 Canola

[00428] For T_1 *AAD -12 (v1)*, 5-6 mg of seed were stratified, sown, and a fine layer of Sunshine Mix # 5 media was added as a top layer of soil. Emerging plants were selected with 560 g ae/ha 2,4-D at 7 and 13 days after planting.

[00429] Surviving plants were transplanted into 3-inch pots containing Metro Mix media. Surviving plants from T1 progenies, that were selected with 560 g ae/ha 2,4-D, were also transplanted into 3-inch pots filled with Metro Mix soil. At 2-4 leaf stage plants were sprayed with either 280, 560, 1120, or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to untransformed control plants. A sampling of T_1 event injury data 14DAT may be seen in **Table 30**. Data suggests that multiple events are robustly resistant to 2240 g ae/ha 2,4-D, while other events demonstrated less robust tolerance up to 1120 g ae/ha 2,4-D. Surviving plants were transplanted to 5 1/4" pots containing Metro Mix media and placed in the same growth conditions as before and self-pollinated to produce only homozygous seed.

Table 30. T_1 *AAD-12 (v1)* and untransformed control response to varying rates postemergence 2,4-D DMA applications.

Herbicide	Untransformed Control	pDAB3759(33)013.001	pDAB3759(18)009.001	pDAB3759(18)022.001	pDAB3759(18)030.001	pDAB3759(18)023.001
	Average % Injury 14DAT					
280 g ae/ha 2,4-D DMA	85	0	0	0	0	0
560 g ae/ha 2,4-D DMA	85	0	0	0	0	0
1120 g ae/ha 2,4-D DMA	90	0	0	13	5	3
2240 g ae/ha 2,4-D DMA	95	1	5	83	31	6

[00430] 22.7-Field tolerance of pDAB3759 canola plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

[00431] Field level tolerance trial was conducted on two *AAD-12 (v1)* events 3759(20)018.001 and 3759(18)030.001 and a wild-type canola (Nex710) in Fowler, Indiana. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Within each herbicide treatment, single 20 ft row/event for event 3759(18)030.0011, 3759(18)018.001 and wild-type line (Nex710) were planted with a 4 row drill on 8 inch row spacing. Herbicide treatments were applied when canola reached the 4-6 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after application.

[00432] Canola response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 31. The wild-type canola (Nex710) was severely injured (72% at 14DAT) by 2,4-D at 2240 g ae/ha which is considered the 4X rate. The *AAD-12 (v1)* events all demonstrated excellent tolerance to 2,4-D at 14DAT with an average injury of 2, 3 and 2% observed at the 1, 2 and 4X rates, respectively. The wild-type canola was severely injured (25% at 14DAT) by the 2X rate of triclopyr (840 g ae/ha). *AAD-12 (v1)* events demonstrated tolerance at 2X rates of triclopyr with an average of 6% injury at 14DAT across the two events. Fluroxypyr at 280 g ae/ha

caused severe injury (37%) to the non-transformed line at 14DAA. *AAD-12 (v1)* events demonstrated increased tolerance with an average of 8% injury at 5DAT.

[00433] These results indicate that *AAD-12 (v1)* transformed events displayed a high level of resistance to 2,4-D, triclopyr and fluroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed canola. AAD-12 has been shown to have relative efficacy of 2,4-D > triclopyr > fluroxypyr.

Table 31. AAD-12 (pDAB3759) canola plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

Herbicide Treatment		% Visual Injury at 14 DAT		
Active Ingredient	Rate	AAD-12 event 3759(20)018.001	AAD-12 event 3759(18)030.001	Wild Type (Nex710)
2,4-D	280 GM AE/HA	0 a	0 b	0 e
2,4-D	560 GM AE/HA	0 a	0 b	15 d
2,4-D	1120 GM AE/HA	2 a	2 ab	33 bc
2,4-D	2240 GM AE/HA	3 a	3 ab	48 a
Triclopyr	840 GM AE/HA	6 a	6 ab	25 cd
Fluroxypyr	280 GM AE/HA	7 a	8 a	37 ab

Means with a column with different letters are significantly different as defined by LSD (p=0.05).

Example 23--*AAD-12 (v1)* Stacked With Insect Resistance (IR) or Other Input Traits in Any Crop

[00434] Insect resistance in crops supplied by a transgenic trait is prevelant in corn and cotton production in North America and across the globe. Commercial products having combined IR and HT traits have been developed by multiple seed companies. These include Bt IR traits (e.g. Bt toxins listed at the website lifesci.sussex.ac.uk, 2006) and any or all of the HTC traits mentioned above. The value this offering brings is the ability to control multiple pest problems through genetic means in a single offering. The convenience of this offering will be restricted if weed control and insect control are accomplished independent of each other. *AAD-12 (v1)* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input traits (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) (isb.vt.edu/cfdocs/fieldtests1.cfm, 2005) either through conventional breeding or jointly as a novel transformation event. Benefits include the convenience and flexibility described in Examples 15-20 above, together with the ability to manage insect pests and/or other

agronomic stresses in addition to the improved weed control offered by *AAD-12* and associated herbicide tolerance. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues.

[00435] Combined traits of IR and HT have application in most agronomic and horticultural/ornamental crops and forestry. The combination of *AAD-12* and its commensurate herbicide tolerance and insect resistance afforded by any of the number of Bt or non-Bt IR genes are can be applied to the crop species listed (but not limited to) in Example 13. One skilled in the art of weed control will recognize that use of any of various commercial herbicides described in Examples 18-20, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by *AAD-12 (v1)* transformation and stacking with the corresponding HT trait or IR trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 24—*AAD-12 (v1)* as an *in vitro* Dicot Selectable Marker

[00436] Genetic engineering of plant cell, tissue, organ, and plant or organelle such as plastid starts with the process of inserting genes of interest into plant cells using a suitable delivery method. However, when a gene is delivered to plant cells, only an extremely small percentage of cells integrate the heterogeneous gene into their genome. In order to select those few cells that have incorporated the gene of interest, researchers link a selectable or screenable "marker gene" to the gene of interest (GOI) in the vector. Cells that contain these markers are identified from the whole population of cells/ tissue to which the DNA plasmid vector was delivered. By selecting those cells that express the marker gene, researchers are able to identify those few cells that may have incorporated the GOI into their genome.

[00437] There are a variety of selectable markers available to enable this selection process to obtain transgenic cells, callus, embryos, shoots and plantlets. The preferred selectable markers by the Ag-industry are herbicide markers that allow the ease of spraying compounds

in the field to select the right transgenic progenies during the process of event sorting in the field situation. *AAD-12 (v1)* has been shown to efficiently serve as a selectable marker for whole plants transformed with the gene in the greenhouse and growth chamber (Example 7) with 2,4-D as the selection agent. Field selection is possible as well using 2,4-D in combination with the *AAD-12 (v1)* gene (Example 11, 22), but use *in* vitro for cell-level selection is complicated by the fact 2,4-D is used almost ubiquitously as a plant growth regulator in the plant tissue culture systems. Degradation of this important hormone by AAD-12 (v1) can impact he ability to use this gene as an in vitro selectable marker. Success of developing 2,4-D as a marker gene depends on identifying the right alternate plant growth regulator that can mimic the effect of 2,4-D in the respective culture system and at the same time possess the ability to be stable and not be degraded by the AAD-12 enzyme when expressed in the transgenic cells. R-dichlorprop is a close analog to 2,4-D that is not a substrate for *AAD-12 (v1)* and is used a non-metabolizable auxin substitute in tobacco cell cultures allowing 2,4-D to be used at high rates as a selection agent. This fact was used in exemplifying *AAD-12 (v1)* could be used as a selectable marker *in vitro*.

[00438] 24.1—Cell culture--Alternative auxins.

[00439] AAD-12 (v1) degrades 2,4-D, but not R-2,4-dichlorophenoxypropionic acid (R-dichlorprop), which has at the same time the structural requirement of an auxinic growth regulator. Other non-metabolizable plant auxin mimics that may be used in cell culture include NAA (naphthalene acetic acid), IAA (indole acetic acid), dicamba, picloram, and R-mecoprop. It was investigated if it was possible to substitute R-dichlorprop and successfully maintain two different tobacco cell cultures PHL (Petite Havanna) and BY2 suspensions. Conversely, for cotton explants R-dichlorprop, dicamba, and picloram were tested as alternative auxins and the embryogenic callus induction response in comparison to the standard growth regulator, 2,4-D was evaluated. Petite Havana tobacco (PHL) and Coker cotton cotyledons were used in their experiments.

[00440] 24.1.1—Tobacco cell suspension—2,4-D as selection agent.

[00441] A dose response study was conducted with both the R-dichlorprop habituated PHL cells and the R-dichlorprop habituated BY2 cells where R-dichlorprop was substituted directly for 2,4-D in culture media. Though the focus was on PHL, a dose response was also done with BY2 in case of possible future studies, as well as to help predict the dose response for PHL. For the dichlorprop habituated PHL dose response, the levels of 2,4-D used (on

LSBY2C medium with R-dichlorprop) were 0 (the control), 1, 2, 3, 5, 8, 10, 12, 15, 18, 20, 40, 60, 80,10,110,120 mg/L 2,4-D. There were four replications per concentration. For the R-dichlorprop habituated BY2 dose response, the levels of 2,4-D used (on LSBY2C medium) were 0 (the control), 1, 2, 3, 5, 8, 10, 20, 30,40, mg/L 2,4-D.

[00442] The dose response was carried out showed that all the concentration of 2,4-D tested were lethal above 10 mg/ L concentrations. However, there was growth in all the concentrations up to 10-mg/L 2,4-D where a slight growth of PHL suspension was observed. The growth of the suspension colonies from 1- 8 mg/L 2,4-D concentrations was comparable to the growth in control treatments. The observation made in BY2 suspension cells were similar except the concentration at 10 mg/L was found to be lethal and the sub lethal concentration was 8 mg/L concentration.

[00443] 24.1.2—Tobacco cell transformation with *AAD-12 (v1)* and 2,4-D selection.

[00444] For tobacco transformation experiment, there were 11 treatments altogether: a control set plated on LS-BY2C + dichlorprop medium , and 10 sets of LSBY2C + dichlorprop + 2,4-D at varying concentration levels (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L). There were four replications per treatment. The plasmid DNA vector used was pDAB724, and the vector used for transformation was EHA101S strain of Agrobacterium tumefaciens. Four ml of PHL suspension at 0.6 $0D^{660}$ were mixed with 100 ul of Agrobacterium (either EHA101 or LBA4404 strains) suspension at 1.0 $0D^{660}$ in a sterile Petri plate and were mixed thoroughly and co-cultivated together in a non-shake condition at a dark growth chamber for 3 days at 25°C . After the co-cultivation period 1.5 ml of the Agro-tobacco suspension mixture was plated to the 11 set of plates above. The experiment was repeated with 13 treatments: a control of LS-BY2C + dichlorprop media (no 2,4-D), and LS-BY2C + dichlorprop + 2,4-D (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L); LSBY2C + 1mg/L 2,4-D + B10 (Bialophos); LSBY2C + 10 2,4-D + B10 + R-dichlorprop. Again, there were four replicates per treatment, as well as a positive and negative control. All media contain 500 mg/L Carbenicillin (C) to control to contain Agrobacterium growth in the selection media.

[00445] The plasmid used in these experiments is pDAB724 and it has *PAT* selectable marker as well. So, control transformation experiments were initiated using R-dichlorprop habituated PHL in the presence of 10 mg/L bialophos following the standard protocol described above. The treatments were done side by side with 4 replicates to see if the bialophos selection in these suspension is normal.

[00446] There was little growth observed in all selection concentrations of 2,4D tested above 10 mg/L; however several fast growing colonies were found in 2, 5, an 8 mg/L 2,4-D concentration and representative sample was transferred to fresh selection at 10 mg/L selection to bulk the callus. Also, several putative colonies were selected in from 12, 15,18 and 20 mg/L 2,4-D, but when compared to 10 mg/L there were only few colonies in these selection plate. Control treatment conducted with bialophos selection showed normal colony development. It appears that 10 mg/L 2,4-D is the sub-lethal and above this concentration 2,4-D appears to be lethal to the non-transformed cells. All the identified colonies were transferred to fresh medium with 10 mg/L selection and were probed for the presence of transgene by PCR as described in Example 10. The colonies selected and bulked had the transgenes as determined by PCR and expression of the genes as established by the Western analyses (as described in example 10). Several colonies were identified as actively growing and transferred to fresh selection medium with 10 mg/L 2,4-D to bulk the callus.

[00447] The bulked calluses were then transferred to higher level of 2,4-D to test the tolerance level *in vitro*. The levels of 2,4-D used were 20, 40, 60, 80, 100, and 120 mg/L 2,4-D. However the callus did not grow beyond 20 mg/L 2, 4-D concentrations indicating a threshold concentration higher than 20 mg/L may exist.

[00448] 24.2.1— Cotton explants—auxin alternatives

[00449] A dose response study was initiated to test multiple auxin alternatives as a substitute for the use of 2,4-D as a growth regulator in cotton. The alternative auxin tested were 2,4-dichlorprop, dicamba, and picloram. These compounds were tested at 0.2, 2.0, and 20.0 uM concentrations respectively. 2,4-D was used as the control treatment at 0.02 uM concentration. The medium used is the base medium for cotton callus induction (Example 12). Beyond the initial phase of culture, auxin is removed from the medium to prod the tissue toward the regeneration process.

[00450] R-dichlorprop was not effective in callus induction of cotyledonary segments and appears toxic to cotton cells at the lowest concentration tested (0.02 uM). Dicamba effectively induces callus growth at all concentrations tested (0.02-20 uM) and has no apparent toxic effects in this concentration range. Callus induction with picloram increased up to a maximum when explants were treated with 0.2 uM to 20 uM. Quality of the callus was consistent with the standard 2,4-D treatment at the 2 uM picloram concentration. At the

highest concentration (20 uM) 2,4-D was also inhibitory to cotton callus generation and growth.

[00451] Cotton has shown initial ability to respond effectively to alternative auxins (to 2,4-D) in culture. At high enough concentrations, 2,4-D is toxic to cotton cotyldeonary explants. R-dichlorprop is surprisingly significantly more toxic to cotton than 2,4-D or other auxins. 2,4-D may be used as a selection agent and in combination with *AAD-12 (v1)* as the selectable marker gene. Other non-metabolizable auxin surrogates (e.g., dicamba, picloram, R-mecoprop, NAA, or IAA) would allow the use of AAD-12 as a selectable marker in dicots with 2,4-D as the selection agent.

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Claims

We claim:

1. A transgenic plant cell comprising a polynucleotide that encodes an AAD-12 protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, a variant of SEQ ID NO:2, and a variant of SEQ ID NO:4, said variant having aryloxyalkanoate dioxygenase activity and at least 95% sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

2. The cell of claim 1 wherein expression of said polynucleotide imparts to said cell an ability to convert a phenoxyacetate auxin herbicide to a non-herbicidal molecule.

3. The cell of claim 1 wherein expression of said polynucleotide imparts to said cell an ability to convert a pyridyloxy auxin herbicide to a non-herbicidal molecule.

4. A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said plant tolerant to said herbicide.

5. A method of selecting for at least one transgenic plant cell of claim 1 wherein said method comprises applying an aryloxyalkanoate herbicide on and/or about said transgenic plant cells and to lacking plant cell that lack said polynucleotide, and allowing said transgenic plant cell and said lacking plant cells to grow.

6. The method of claim 5 wherein said transgenic plant cell is a plant cell of a transgenic crop plant growing in a field, and said lacking cells are cells of a weed growing in said field.

7. The method of claim 5 wherein said transgenic plant cell is a plant cell transformed with said polynucleotide, and said lacking cells are non-transformants, and all of said cells are grown on tissue culture medium.

8. A method of controlling weeds, said method comprising applying a 2,4-D herbicide to a crop plant and to a weed, said crop plant comprising a plurality of plant cells according to claim 1.

9. The method of claim 8, wherein said crop plant is a soybean plant.

10. The method of claim 8, wherein said weed is resistant to glyphosate.

11. A method of protecting a crop plant from damage by an aryloxyalkanoate dioxygenase herbicide, said method comprising growing a plant comprising an AAD-12 gene, and applying said herbicide to said plant.

12. The method of claim 11 wherein said herbicide is 2,4-D.

13. The method of claim 8 wherein said plant further comprises a glyphosate resistance gene, and said method further comprises applying glyphosate to said plant and to said weed.

14. The method of claim 13, said plant further comprising a third herbicide resistance gene, and said method further comprising applying a third herbicide to said plant and to said weed.

15. A transgenic plant comprising a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide.

16. The transgenic plant of claim 15, said polynucleotide also conferring resistance to a phenoxy auxin herbicide.

17. The plant of claim 16, said phenoxy auxin herbicide selected from the group consisting of 2,4-D and MCPA.

18. The plant of claim 15, said plant further comprising at least one additional herbicide resistance gene.

19. A plant comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

20. A polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said polynucleotide comprises a codon composition that is optimized for expression in a plant.

21. A plant cell comprising a heterologous polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

22. A method of selecting for a transformed plant cell of claim 21 wherein said method comprises subjecting a plurality of plant cells to transformation with said polynucleotide, then growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin herbicide.

23. The method of claim 22 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

24. A method of controlling at least one weed in a field, said method comprising planting in said field seed of at least one transgenic plant, said plant comprising

a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide, and

a second heterologous polynucleotide encodes an enzyme conferring resistance to at least one other herbicide;

said method further comprising applying to at least a portion of said field a first herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide; and applying said at least one other herbicide to said at least a portion of said field.

25. The method of claim 24 wherein said herbicides are applied sequentially or concurrently.

26. The method of claim 24 wherein said first herbicide is a phenoxy auxin herbicide.

27. The method of claim 24 wherein said at least one other herbicide is selected from the group consisting of acetochlor, acifluorfen, alloxydim, amidosulfuron, aminopyralid, atrazine, beflubutamid, bispyribac, butafenacil, cafenstrole, carfentrazone, chlorimuron, chlorotoluron, cinidon-ethyl, clethodim, clodinafop, clomazone, cloproxydim, clopyralid, cloransulam, cyanazine, cyclosulfamuron, cycloxydim, cyhalofop, daimuron, dicamba, diclofop, diclosulam, diflufenican, dimethenamid, diquat, dithiopyr, diuron, ethalfluralin, fenoxaprop, flazasulfuron, florasulam, fluazifop, flucarbazone, flufenacet, flufenican, flufenpyr, flumetsulam, flumiclorac, flumioxazin, fluroxypyr, fluthiacet, fomesafen, foramsulfuron, glufosinate, glyphosate, halosafen, halosulfuron, haloxyfop, imazamethabenz, imazamox, imazapic, imazapyr, imazaquin, imazethapyr, imazosulfuron, iodosulfuron, ioxynil, isoxaben, isoxaflutole, lactofen, linuron, mefenacet, mefluidide, mesosulfuron, mesotrione, metamifop, metazachlor, metosulam, metribuzin, MSMA, napropamide, nicosulfuron, norflurazon, oryzalin, oxadiazon, oxyfluorfen, paraquat, pebulate, pendimethalin, penoxsulam, picloram, picolinafen, pinoxaden, primisulfuron, profoxydim, propanil, pyraflufen, pyrazosulfuron, pyribenzoxim, pyriminobac, pyrithiobac, pyroxasulfone, pyroxsulam, quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxydim, terbacil, thiazopyr, thidiazuron, thiencarbazone, thifensulfuron, thiobencarb, topramezone,

tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflusulfuron, and tritosulfuron.

28. A method of assaying a protein for its ability to cleave a pyridyloxyalkanoate herbicide, said method comprising providing a sample comprising said protein and a pyridyloxy auxin herbicide, and assaying said sample for presence of a chloropyridinol.

29. A method of assaying a protein for aryloxyalkanoate dioxygenase activity wherein said method comprises providing a sample comprising said protein and 2-(2-chloro,4-nitrophenoxy) propionate and/or acetate, and assaying said sample for presence of a 2-chloro,4-nitrophenol.

30. A heritable expression cassette comprising a nucleic acid molecule encoding a protein that enzymatically degrades at least one herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein said nucleic acid molecule hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

31. The expression cassette of claim 30 wherein said promoter is a plant promoter.

32. The expression cassette of claim 30 comprising a promoter selected from a cassava vein mosaic virus promoter, CaMV 35S promoter, Figwort Mosaic Virus promoter, rice actin promoter, phaseolin promoter, *Arabidopsis thaliana* Ubiquitin 10 promoter, maize ubiquitin promoter, *Arabidopsis thaliana* Act2 promoter, *Arabidopsis thaliana* Ubiquitin 11 promoter, and *Arabidopsis thaliana* Ubiquitin 3 promoter.

33. The method of claim 24 wherein said plant is resistant to a herbicide formulation comprising glyphosate.

34. A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said

first herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin.

35. The method of claim 34 wherein said first herbicide is an acid, an inorganic salt, an organic salt, or an ester.

36. The method of claim 34 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

37. The method of claim 36 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate.

38. A method of detecting whether a plant comprises a polynucleotide of claim 20 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

39. The method of claim 38 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

40. The method of 38 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

41. The method of 39 wherein said method comprises using an antibody to detect for the presence of said protein.

42. The plant of claim 15 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

43. The plant of claim 15 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance,

increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

44. A method of controlling at least one weed in a field, said method comprising growing at least one transgenic plant of claim 15 in said field, and applying a pyridyloxy auxin herbicide to at least a portion of said field.

45. A method of controlling at least one weed in a field, said method comprising growing at least one plant of claim 16 in said field, and applying a phenoxy auxin herbicide to at least a portion of said field.

46. The method of claim 44, further comprising applying a phenoxy auxin herbicide to at least a portion of said field.

47. The method of claim 44, said pyridyloxy auxin herbicide selected from the group consisting of fluroxypyr and triclopyr.

48. The method of claim 45, said phenoxy auxin herbicide selected from the group consisting of 2,4-D and MCPA.

49. The method of claim 45 wherein said plant is resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, imazethapyr, chlorsulfuron, dicamba, mesotrione, isoxaflutole, and butafenacil.

50. The method of claim 45 wherein said herbicide is an achiral phenoxy auxin.

51. The method of claim 24 wherein said plant is a monocot.

52. The method of claim 51 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, warm and cool-season turf grass, oats, sorghum, and pasture grasses.

53. The method of claim 24 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

54. The method of claim 53 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

55. A method of conferring herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a polynucleotide encoding an enzyme capable of using a pyridyloxy auxin herbicide and a phenoxy auxin herbicide as a substrate.

56. A method of controlling glyphosate-resistant, naturally glyphosate tolerant, and/or naturally glufosinate tolerant weeds in a field of glyphosate and/or glufosinate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 13, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

57. The method of claim 56 wherein said herbicide is a phenoxy auxin.

58. The method of claim 57 wherein said phenoxy auxin is 2,4-D.

59. A method of controlling weeds that are resistant to an ALS-inhibiting herbicide in a field of crop plants that comprise a polynucleotide of claim 20, said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

60. The cell of claim 1 wherein said plant cell is selected from the group consisting of dicotyledonous cells and monocotyledonous cells.

61. The cell of claim 60 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

62. The cell of claim 60 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

63. The cell of claim 61 wherein said pyridyloxy auxin herbicide is selected from the group consisting of fluroxypyr and triclopyr.

64. The plant of claim 4 wherein expression of said polynucleotide renders said plant resistant to both a phenoxyacetate auxin herbicide and a pyridyloxy auxin herbicide.

65. The method of claim 8 wherein said method comprises applying a second herbicide.

66. The method of claim 22 wherein said 2,4-D herbicide and said second herbicide are applied sequentially.

67. The method of claim 22 wherein said first herbicide and said second herbicide are applied concurrently.

68. The method of claim 8 wherein said plant is resistant to glyphosate.

69. The method of claim 24 wherein said second polynucleotide is a modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase).

70. The method of claim 24 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

71. The method of claim 70 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

72. The method of claim 24 wherein said method further comprises applying a third herbicide.

73. A polynucleotide optimized for expression in a plant wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

74. The polynucleotide of claim 44 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

75. An isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell.

76. The polynucleotide of claim 75 wherein said promoter is a plant promoter.

77. The polynucleotide of claim 76 wherein said promoter is a cassava vein mosaic virus promoter.

78. The method of claim 22 wherein said polynucleotide is used as a selectable marker.

79. A seed comprising a plant cell of claim 1.

80. A plant grown from the seed of claim 79.

81. A regenerable part, progeny, or asexual propagate of the plant of claim 4.

82. The method of claim 8 wherein said method is used to treat or prevent herbicide-resistant weeds.

83. The plant of claim 4 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

84. The plant of claim 4 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

85. A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 20, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

86. The method of claim 85 wherein said herbicide is a phenoxy auxin.

87. The method of claim 25 wherein said herbicides are applied from a tank mix.

88. The method of claim 10 wherein said at least one of said weeds comprises at least one glyphosate resistant volunteer plant of a different species than said transgenic crop.

1/3

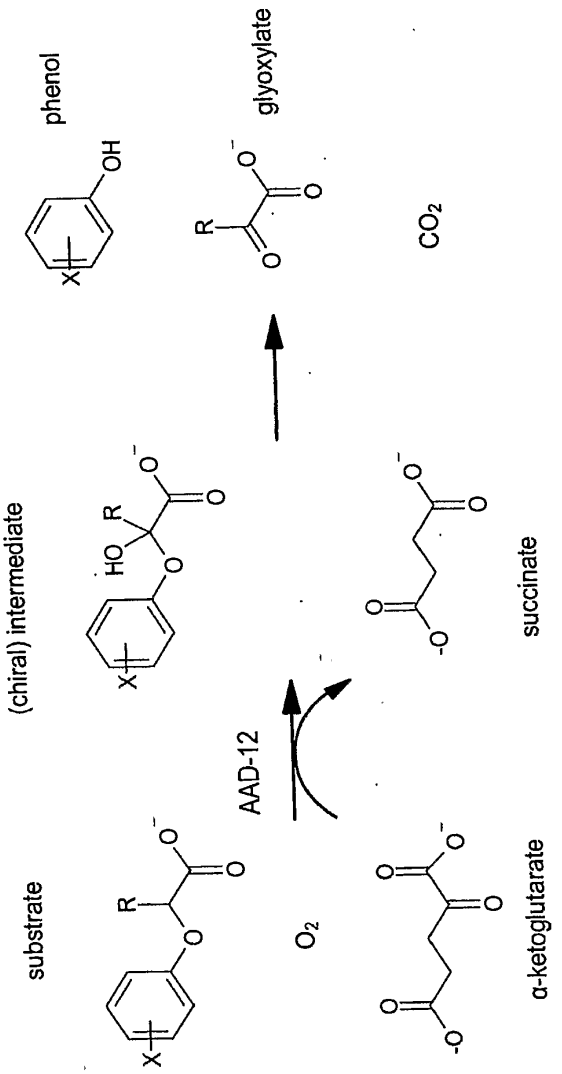


FIG. 1

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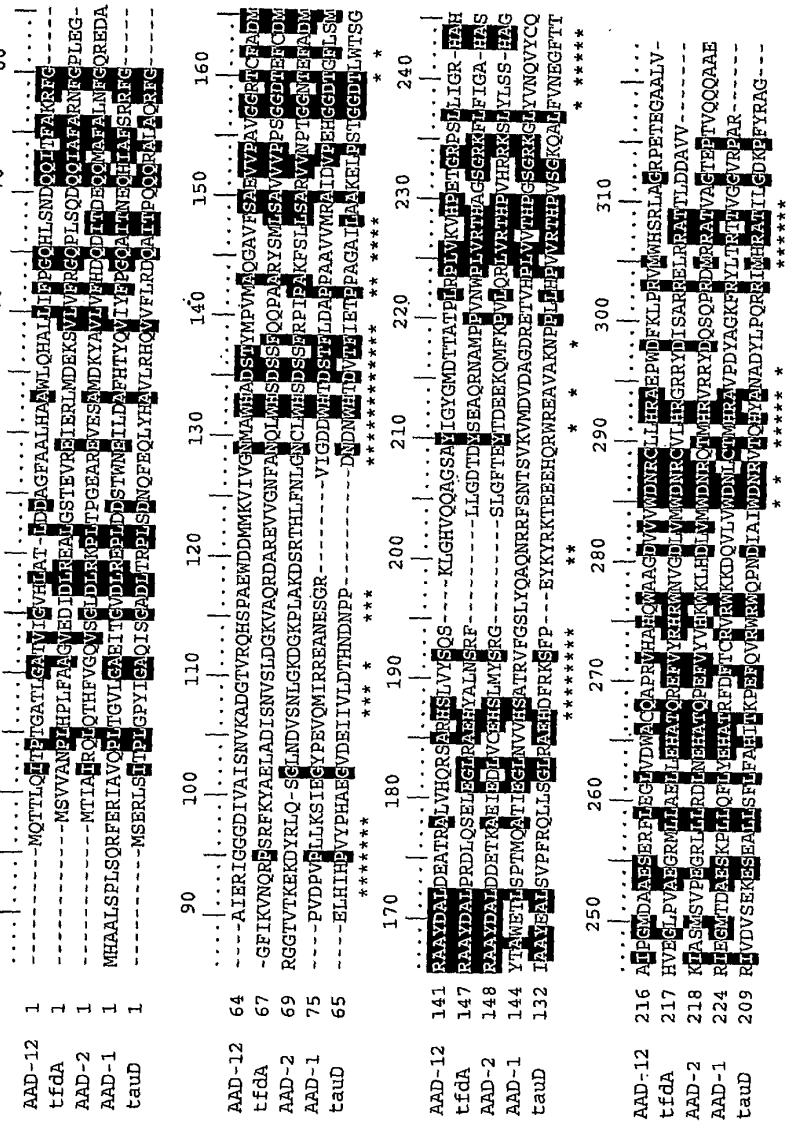


FIG. 2

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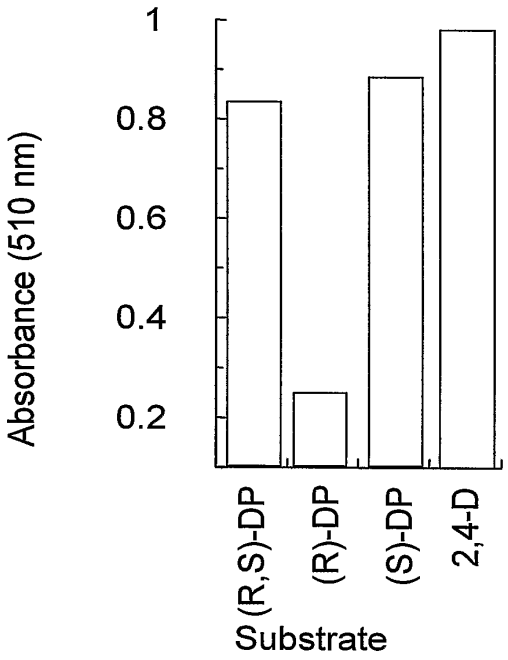


FIG. 3

SEQUENCE LISTING

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Merlo, Donald J.
Jayakumar, Pon Samuel
Lin, Gaofeng

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World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

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FILING DATE: *October 28, 2005*
RELATED PCT APPLICATION NUMBER: *PCT/US06/42133*

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This is a request for filing PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EQ 005884833 US

112963 U.S. PTO
60/731044
102805

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Justin M.	Lira	Indianapolis, IN
Terence A.	Walsh	Zionsville, IN
Donald J.	Merlo	Carmel, IN

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max):

Novel Herbicide Resistance Genes

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☒ Customer Number 23557

OR

☐ Firm or Individual Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS ***(check all that apply)***

☐ Application Data Sheet. See 37 CFR 1.76
☒ Specification *Number of Pages* 117
☒ Drawing(s) *Number of Sheets* 1
☐ CD(s), Number of CDs _____
☒ Other (specify) Sequence Listing (3 pages)

Fees Due: Filing Fee of $200 ($100 for small entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is due, which is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

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☐ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fee and application size fee (if applicable).
☐ Payment by credit card. Form PTO-2038 is attached.

TOTAL FEE AMOUNT ($)
$450.00

☒ The Director is hereby authorized to charge the filing fee and application size fee (if applicable) or credit any payment to Deposit Account Number: 19-0065 . A duplicative copy of this form is enclosed for fee processing.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

SIGNATURE _____

Date October 28, 2005

REGISTRATION NO. 39,355

TYPED or PRINTED NAME Jay M. Sanders

Docket Number: DAS-129P

TELEPHONE (352) 375-8100

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This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Provisional Patent Application
Docket No. DAS-129P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. : DAS-129P

Applicant(s) : Terry R. Wright, Justin M. Lira, Terry Walsh, Donald J. Merlo

For : Novel Herbicide Resistance Genes

COMMISSIONER FOR PATENTS
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Express Mail No.. EQ005884833US Date of Deposit: October 28, 2005

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Drew Core
Typed/Printed Name of Person Mailing Paper

Signature

J:\DAS\DAS-129P\Application\Cert of Mailing.doc/DNB/bra

NOVEL HERBICIDE RESISTANCE GENES

Background of the Invention

[0001] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0002] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

[0003] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (*e.g.*, wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (*e.g.*, alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; *see* "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0004] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

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[0005] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Ng *et al.*, 2003; Simarmata *et al.*, 2003; Lorraine-Colwill *et al.*, 2003; Sfiligoj, 2004; Miller *et al.*, 2003; Heap, 2005; Murphy *et al.*, 2002; Martin *et al.*, 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis,* and *Plantago lanceolata.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0006] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

[0007] 2,4-D is in the phenoxy acid class of herbicides, as is MCPA. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf

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weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known. Triclopyr and fluroxypyr are pyridyloxyacetic acid herbicides whose mode of action is as a synthetic auxin, also.

[0008] These herbicides have different levels of selectivity on certain plants (*e.g.*, dicots are more sensitive than grasses). Differential metabolism by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

[0009] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D currently formulated has a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0010] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0011] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-

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ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0012] One unique example with low homology to *tfdA* (31% amino acid identity) is *sdpA* from *Delftia acidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002, Westendorf *et al., 2003*). This enzyme has been shown to catalyze the first step in (S)-dichlorprop (and other (S)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Transformation of this gene into plants, has not heretofore been reported.

[0013] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0014] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxyacetate auxins (such as 2,4-D and dichlorprop), pyridyloxyacetate auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. A multifunctional enzyme for the degradation of herbicides covering multiple modes of action has recently been described (PCT US/2005/014737; filed May 2, 2005). Another unique multifunctional enzyme and potential uses are described hereafter.

Brief Summary of the Invention

[0015] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, aryloxyalkanoate-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more

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robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0016] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, MCPA, triclopyr, fluroxypyr, and one or more commercially available herbicides (*e.g.*, glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0017] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide-tolerant crop (HTC) trait and selectable marker opportunities.

[0018] There was no prior motivation to produce plants comprising an *AAD-12* gene (preferably an *AAD-12* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-12 enzyme to render the plants resistant to not only phenoxyacetic acid herbicides (such as 2,4-D) but also the pyridyloxyacetates herbicides,

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triclopyr and fluroxypyr. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0019] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxyacetate auxin and pyridyloxyacetates auxin herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid and other aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more pyridyloxyacetate or phenoxyacetate auxin herbicides to plants comprising an *AAD-12* gene. The subject invention also provides methods of using an *AAD-12* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-12*, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0020] **Figure 1** illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop.

Brief Description of the Sequences

[0021] **SEQ ID NO:1** is the nucleotide sequence of *AAD-12* from *Delftia acidovorans*.

[0022] **SEQ ID NO:2** is the translated protein sequence encoded by SEQ ID NO:1.

[0023] **SEQ ID NO:3** is the plant optimized nucleotide sequence of *AAD-12 (v1)*.

[0024] **SEQ ID NO:4** is the translated protein sequence encoded by SEQ ID NO:3.

[0025] **SEQ ID NO:5** is the E.coli optimized nucleotide sequence of *AAD-12 (v2)*.

[0026] **SEQ ID NO:6** is the sequence of the M13 forward primer.

[0027] **SEQ ID NO:7** is the sequence of the M13 reverse primer.

[0028] **SEQ ID NO:8** is the sequence of the forward PTU primer.

[0029] **SEQ ID NO:9** is the sequence of the reverse PTU primer.

[0030] **SEQ ID NO:10** is the sequence of the *AAD-12 (v1)* PTU forward PCR primer.

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[0031] **SEQ ID NO:11** is the sequence of the *AAD-12 (v1)* PTU reverse PCR primer.

[0032] **SEQ ID NO:12** shows the sequence of the "NcoI of Brady" primer.

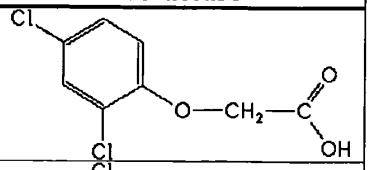
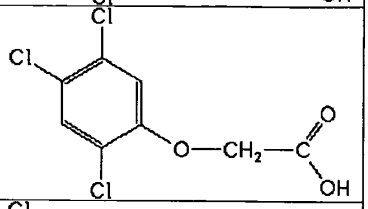
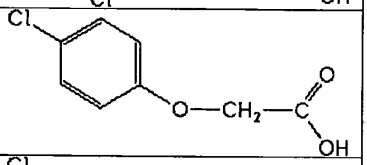
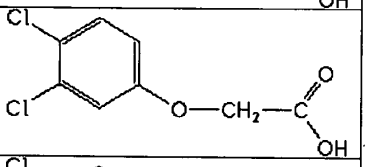
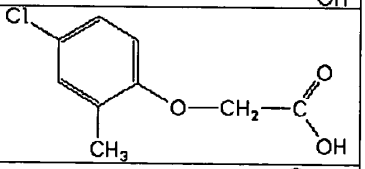
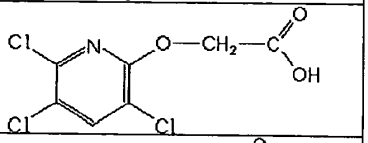
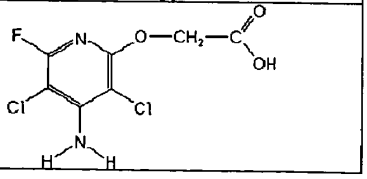
[0033] **SEQ ID NO:13** shows the sequence of the "SacI of Brady" primer.

Detailed Description of the Invention

[0034] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the *AAD-12* gene is that unlike all *tfdA* homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (*e.g.,* triclopyr, fluroxypyr) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). *See* **Table 1**. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the *AAD-12* gene in plants would afford protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled, protecting from drift or other off-site synthetic auxin herbicide injury for the full breadth of commercially available phenoxy auxins. **Table 1** defines commercially available pyridyl and phenoxy auxins and provides relevant chemical structures.

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Table 1. Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	
MCPA	94-74-6	25 – 4000	125 - 1550	
Triclopyr	55335-06-3	50 – 2000	70 – 840	
Fluroxypyr	69377-81-7	25 – 2000	35 – 560	

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[0035] A single gene (*AAD-12*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-12* can provide protection *in planta* to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing *AAD-12* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

[0036] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-12* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (*e.g.*, 2,4-D) and pyridyloxyacetates auxin herbicides (*e.g.*, triclopyr)—selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0037] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the

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ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/*AAD-12* stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0038] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0039] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS, GOX, GAT*), glufosinate resistance (*e.g.*, *Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g.*, *PPO-1*), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), dicamba-degrading enzymes (*see, e.g.*, US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

[0040] Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione,

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isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0041] Additionally, *AAD-12* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0042] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-1 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

[0043] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0044] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and pyridyloxyacetate herbicides. Thus, the subject invention relates in part to the degradation of

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2,4-dichlorophenoxyacetic acid, other phenoxyacetic acids, and pyridyloxyacetic acid herbicides by a recombinantly expressed AAD-12 enzyme.

[0045] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP *in vitro*. An additional advantage that *AAD-12* transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0046] The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an *AAD-12* gene.

[0047] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0048] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0049] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-

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ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0050] The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, *sdpA*, from *Delftia acidivorans* (Westendorf *et al.*, 2002, 2003) with low homology to *tfdA* (31% amino acid identity). This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. *SdpA* has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0051] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

[0052] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and pyridyloxy auxin herbicides.

[0053] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. *AAD-12* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (*e.g.*, glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, *et al.*), and insect resistance traits (*Cry1F, Cry1Ab, Cry 34/45, et al.*) for example.

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Additionally, *AAD-12* can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0054] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-12*-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention.

[0055] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[0056] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

[0057] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[0058] The transgenic expression of the subject *AAD-12* genes is exemplified in, for example, *Arabidopsis*, tobacco, soybean, and canola. However, the subject invention can be applied to any other desired types of plants, such as corn (maize), cotton, and rice. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as warm season turf grass) and dicot crops. Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in

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grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

[0059] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

[0060] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

[0061] Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that

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the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[0062] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

[0063] The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[0064] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[0065] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as

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genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the order Proteobacteria, and specific genera with known *sdpA*'s, such as *Sphingobium* and *Delftia*, for example, can be used as source isolates.

[0066] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[0067] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[0068] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook *et al.*, 1989, which is incorporated herein by reference.

[0069] Polynucleotides and probes. The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[0070] The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control

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of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-12* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

[0071] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[0072] In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

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[0073] Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

[0074] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

[0075] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl

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(175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[0076] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[0077] Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

[0078] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

$$Tm = 81.5° C + 16.6 \text{ Log}[Na+] + 0.41(\%G+C) - 0.61(\%\text{formamide}) - 600/\text{length of duplex in base pairs.}$$

[0079] Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

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[0080] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

Tm (°C) = 2(number T/A base pairs) + 4(number G/C base pairs)

(Suggs *et al.*, 1981).

[0081] Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

[0082] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low:	1 or 2x SSPE, room temperature
Low:	1 or 2x SSPE, 42° C
Moderate:	0.2x or 1x SSPE, 65° C
High:	0.1x SSPE, 65° C.

[0083] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[0084] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.*, 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the

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primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[0085] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[0086] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be

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made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[0087] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[0088] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[0089] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226,

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227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, or 293 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[0090] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[0091] It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.*, Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term "protein" can include functionally active truncations.

[0092] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62,

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63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[0093] Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[0094] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.*, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[0095] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within

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the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 2** provides a listing of examples of amino acids belonging to each class.

Table 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[0096] In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

[0097] As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

[0098] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

[0099] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or

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monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

[00100] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[00101] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[00102] Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[00103] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[00104] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression

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of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[00105] Vectors comprising an *AAD-12* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc.* Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli.* The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

[00106] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria.* The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria.*

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They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[00107] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00108] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[00109] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (*PAT*), glyphosate (*EPSPS*), imazethyapyr (*AHAS)*, and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins,

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thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00110] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. *See* U.S. Patent No. 6,809,232. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00111] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the

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construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[00112] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00113] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

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[00114] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00115] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

[00116] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00117] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like

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may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00118] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00119] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are

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described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00120] Unless specifically indicated or implied, the terms "a", "an", and "the" signify "at least one" as used herein.

[00121] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00122] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Method for Identifying Genes That Impart Resistance to 2,4-D *In Planta*

[00123] As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (*i.e.*, α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with ≤ 50% homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to *tfdA* from *Ralstonia eutropha)* can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00124] A single gene (*sdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 31% amino acid

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identity to *tfdA*. Percent identity was determined by first translating both the *sdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

Example 2 – Optimization of Sequence for Expression in Plants and Bacteria

[00125] 2.1 – Background.

[00126] To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[00127] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00128] **Table 3** illustrates how high the G+C content is in maize. For the data in **Table 3**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

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Table 3: Compilation of G + C contents of protein coding regions of maize genes

Protein Class[a]	Range % G + C	Mean % G + C[b]
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)[c]

[a] Number of genes in class given in parentheses.
[b]Standard deviations given in parentheses.
[c] Combined groups mean ignored in mean calculation

[00129] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[00130] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 4**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to

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specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, *etc.* with the next preferred codon of choice.

Table 4. Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

[00131] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first

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choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00132] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

[00133] 2.2 – *AAD-12* Plant rebuild analysis.

[00134] Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native *AAD-12* coding region (SEQ ID NO:1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 (AAD-12) is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence *AAD-12 (v1)* (SEQ ID NO:3) was developed that encodes a protein (SEQ ID NO:4) which is the same as the native SEQ ID NO:2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO:4). The additional alanine codon (GCT; underlined in SEQ ID NO:3) encodes part of an *Nco*I restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical. **Table 5** shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences

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(Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

Table 5. Codon composition comparisons of coding regions of Native AAD-*12*, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #	Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #
ALA (A)	GCA	1	10	11	LEU (L)	CTA	0	0	0
	GCC	35	16	15		CTC	1	8	8
	GCG	7	0	0		CTG	23	0	0
	GCT	0	18	17		CTT	0	8	8
ARG (R)	AGA	0	4	5		TTA	0	0	0
	AGG	0	4	6		TTG	0	8	8
	CGA	0	0	0	LYS (K)	AAA	1	1	2
	CGC	15	6	4		AAG	5	5	4
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	4	3	PHE (F)	TTC	7	5	5
ASN (N)	AAC	3	2	2		TTT	1	3	3
	AAT	1	2	2	PRO (P)	CCA	0	5	6
ASP (D)	GAC	15	9	9		CCC	9	4	4
	GAT	2	8	8		CCG	5	0	0
CYS (C)	TGC	3	2	2		CCT	0	5	5
	TGT	0	1	1	SER (S)	AGC	5	4	3
END	TAA	1	0	1		AGT	0	0	0
	TAG	0	0			TCA	0	3	3
	TGA	0	1			TCC	2	3	3
GLN (Q)	CAA	1	8	7		TCG	6	0	0
	CAG	13	6	7		TCT	0	3	3
GLU (E)	GAA	3	4	4	THR (T)	ACA	1	4	5
	GAG	8	7	7		ACC	11	7	7
GLY (G)	GGA	0	8	7		ACG	5	0	0
	GGC	24	7	7		ACT	1	7	6
	GGG	1	3	4	TRP (W)	TGG	8	8	8
	GGT	0	7	7	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	9	9		TAT	1	2	2
	CAT	8	7	7	VAL (V)	GTA	0	0	0
ILE (I)	ATA	0	2	2		GTC	6	8	7
	ATC	10	4	5		GTG	18	8	9
	ATT	1	5	4		GTT	0	8	8
	Totals	163	164	163		Totals	130	130	130

[00135] It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

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[00136] 2.3 Rebuild for *E. coli* Expression

[00137] Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in *Escherichia coli and Salmonella typhimurium cellular and molecular biology, vol. 2*, pp. 2047–2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umbarger, H. (eds.) American Society for Microbiology, Washington, DC). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for these *E. coli*-Class II gene coding regions. It is thought that a protein coding region having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO:4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (*i.e.* Shine Dalgarno sequences) The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO:5 and encodes the protein disclosed in SEQ ID NO:4.

[00138] The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. **Table 6A** presents the codon compositions of the native *AAD-12* coding region (Columns A and D), an *AAD-12* coding region optimized for expression in *E. coli* (v3; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).

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Table 6A. Codon composition comparisons of coding regions of Native *AAD-12*, *E. coli*-Optimized version (v2) and a Theoretical *E. coli* Class II-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor. Class II #	Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor, Class II #
ALA (A)	GCA	1	13	13	LEU (L)	CTA	0	0	0
	GCC	35	0	0		CTC	1	2	0
	GCG	7	18	17		CTG	23	20	24
	GCT	0	13	14		CTT	0	1	0
ARG (R)	AGA	0	0	0		TTA	0	1	0
	AGG	0	0	0		TTG	0	0	0
	CGA	0	0	0	LYS (K)	AAA	1	4	5
	CGC	15	6	6		AAG	5	2	1
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	12	12	PHE (F)	TTC	7	6	6
ASN (N)	AAC	3	4	4		TTT	1	2	2
	AAT	1	0	0	PRO (P)	CCA	0	3	2
ASP (D)	GAC	15	10	9		CCC	9	0	0
	GAT	2	7	8		CCG	5	11	12
CYS (C)	TGC	3	2	2		CCT	0	0	0
	TGT	0	1	1	SER (S)	AGC	5	4	4
END	TAA	1	1	1		AGT	0	0	0
	TAG	0	0	0		TCA	0	0	0
	TGA	0	0	0		TCC	2	5	4
GLN (Q)	CAA	1	3	3		TCG	6	0	0
	CAG	13	11	11		TCT	0	4	5
GLU (E)	GAA	3	8	8	THR (T)	ACA	1	0	0
	GAG	8	3	3		ACC	11	12	12
GLY (G)	GGA	0	0	0		ACG	5	0	0
	GGC	24	12	11		ACT	1	6	6
	GGG	1	0	0	TRP (W)	TGG	8	8	8
	GGT	0	13	14	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	11	11		TAT	1	2	2
	CAT	8	5	5	VAL (V)	GTA	0	6	6
ILE (I)	ATA	0	0	0		GTC	6	0	0
	ATC	10	7	7		GTG	18	8	7
	ATT	1	4	4		GTT	0	10	11
	Totals	163	164	164		Totals	130	130	130

[00139] It is clear from examination of **Table 6A** that the native and *E. coli*-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

[00140] 2.4 – Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence

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of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO:20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00141] A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in **Table 6B**. Columns D and H of **Table 6B** present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in **Table 6B.** Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

Weighted % of C1 =1/(%C1 + %C2 + %C3 + *etc.*) x %C1 x 100

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of **Table 6B** (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of **Table 6B**. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

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Table 6B. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	Codon	Weighted %	Soybean %	Amino Acid	Codon	Weighted %	Soybean %
ALA (A)	GCA	33.1	30.3	LEU (L)	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	LYS (K)	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	**6.0**	MET (M)	ATG	100.0	100
	CGT	16.9	14.5	PHE (F)	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	PRO (P)	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	SER (S)	AGC	16.0	15.1
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	**6.1**
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	THR (T)	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	**8.3**
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	TRP (W)	TGG	100.0	100
	GGT	30.4	30.4	TYR (Y)	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	VAL (V)	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

[00142] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 from PCT/US2005/014737 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the

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numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00143] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is disclosed as bases 1-1575 of SEQ ID NO:22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO:22 of PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston TX).

Example 3-Cloning of expression and transformation vectors

[00144] 3.1 Construction of E.coli, pET expression vector.

[00145] Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba 1, Xho 1) *AAD-12 (v2)* was cut out of the picoscript vector X and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

[00146] To differentiate between *AAD-12 (v2)*:pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C for 4 hours with agitation. Each culture was then spotted onto LB K plates, which were incubated at 37° C overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3222.

[00147] 3.2- Construction of *Pseudomonas* expression vector

[00148] The *AAD-12 (v2)* open reading frame was initially cloned into the modified pET expression vector (Novagen), "pET280 S/S", as an XbaI – XhoI fragment. The resulting

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plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The AAD12 open reading frame from pDAB725 was transferred into the *Pseudomonas* expression vector, pMYC1803, as an XbaI – XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB321 *Pseudomonas* expression strains.

[00149] 3.3 – Completion of binary vectors.

[00150] The plant optimized gene *AAD-12 (v1)* was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:6) and M13 Reverse (SEQ ID NO:7) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-12 (v1)* DNA sequence. The *AAD-12 (v1)* gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-12 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB724, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-12 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium* (see section 7.2).

[00151] 3.4 - Cloning of additional transformation constructs.

[00152] All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular cloning methods (Maniatis *et al.,* 1982). **Table 7** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

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Table 7. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
724		A, Ct, S	AAD12 v1	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3274		A	AAD12 v1	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	-	-	Agro binary
3277		S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2			Spectinomycin		pat	AtUbi10	Agro binary
3278	1580	T	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2			Spectinomycin	-	pat	AtUbi10	Agro binary
3285		A	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3759	-	A, Ca, S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi10	Agro binary
4101		Cn, R	AAD12 v1	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers / Gun

*A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region

(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

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Example 4 - Recombinant AAD-12 (v2) Expression in *Pseudomonas fluorescens*

[00153] 4.1- *Pseudomonas fluorescens* culture

[00154] 200 µl of *Pseudomonas fluorescens* glycerol stock carried plant-optimized *AAD-12* gene (v2) was used to inoculate 50 ml fresh LB media supplemented with 30 µg/ml tetracycline/HCl. The culture (in a 250 ml baffled Erlenmeyer flask) was incubated on a shaker (New Brunswick Scientific Model Innova 44) at 300 rpm and 30 °C for 16 hrs. 20 ml of seed culture was transferred into 1 L *Pseudomonas fluorescens* culture media (Yeast extract, 5 g/L; K_2HPO_4, 5 g/L; $(NH_4)_2PO_4$, 7.5 g/L; $(NH_4)_2SO_4$; $MgSO_4$-$7H_2O$, 1 g/L; KCl, 0.5 g/L; $CaCl_2$-$2H_2O$, 0.5 g/L; NaCitrate-$2H_2O$, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: $FeCl_3$-$6H_2O$, 5.4 g/L; $MnCl_2$-$4H_2O$, 1 g/L; $ZnSO_4$-$7H_2O$, 1.45 g/L; $CuSO_4$-$5H_2O$, 0.25 g/L; H_3BO_3, 0.1 g/L; $(NH_4)_6MO_7O_{24}$, 0.1 g/L; concentrated HCl, 13 ml/L) supplemented with 20 µg/ml tetracycline/HCl and 250 µl of anti-form Pluronic L61 in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 30°C and 300 rpm for 24 hrs. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM final in the cultures and continued to incubate for 48 hrs at same conditions. Cells were harvested by centrifugation at 7 krpm at 4 °C for 15 min, and cell paste was stored at -80 °C or immediately processed for purification.

[00155] 4.2- Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

[00156] Frozen or fresh *Pseudomonas* cells from 6 L culture were thawed and resuspended in approximately 1000 ml of extraction buffer containing 20 mM Tris-HCl, pH 8.5, and 25 ml of Protease Inhibitor Cocktail (Sigma cat# P8465), and disrupted on ice for 20 minutes by sonication. The lysate was centrifuged at 24,000 RPM for 20 min. The supernatant was transferred, and dialyzed against 5 L of 20 mM Tris-HCl, pH 8.5 overnight at 4 °C, and applied at 15 ml/min to a Q Sepharose Fast Flow column (Pharmacia XK 50/00, 500 ml bed size) equilibrated with the same buffer. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4 °C. The column was washed with this buffer until the eluate OD_{280} returned to baseline, then proteins were eluted with 2 L of linear gradient from 0 to 0.2 M NaCl at a flow rate of 15 ml/min, while 45 ml fractions were collected. Fractions containing AAD-12 activity as determined by the colorimetric enzyme assay and also corresponding to the predicted molecular weight of AAD-12 protein (about 32 kDa band on SDS-PAGE) were pooled. Solid ammonium sulfate to final 0.5 M was added to

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the sample, and then applied to a Phenyl Source 15 column (Pharmacia HR 16/10, 20 ml bed size) equilibrated in 0.5 M ammonium sulfate in 20 mM Tris-HCl, pH 8.5. This column was washed with the binding buffer at 4 ml/min until the OD_{280} of the eluate returned to baseline, then proteins were eluted within 50 min (4 ml/min) by a linear gradient from 0.5 M to 0 Ammonium sulfate in 20 mM Tris-HCl, pH 8.5, and 4 ml fractions were collected. The main peak fractions contain AAD-12 was pooled, concentrated to 5 ml using MWCO 10 kDa membrame centrifugal filter device (Millipore). The sample was then applied to a Superdex 75 gel filtration column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure AAD-12 were pooled and stored at -80 °C. Protein concentration was determined by Bradford assay or total amino acid analysis using bovine serum albumin as standard.

[00157] Ten milligrams purified AAD-12 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

Example 5 – *In vitro* assays of AAD-12 activity

[00158] 5.1 – Assay via colorimetric phenol detection.

[00159] Enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 µM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 µM $NH_4(FeSO_4)_2$, 200 µM sodium ascorbate. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol was linear and proportional to the concentration of phenol in the assay up to ~500 µM. A calibration curve

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performed under standard assay conditions (160 µl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2 µM phenol.

[00160] Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200 µM NH_4FeSO_4, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 5 minutes of incubation at 25° C, the reaction was terminated by addition of 30 µl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH) and 0.2 % 4-aminoantipyrine. Then 10 µl 0.8 % potassium ferricyanide was added and after 5 or 10 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols.

[00161] 5.2 – Assay via detection of chloropyridinol

[00162] AAD-12 action on potential substrates such as the herbicide triclopyr containing a substituted pyridine (rather than benzene rings) will release a pyridinol on cleavage of the aryloxyalkanoate bond. Pyridinols were not detected using the aminoantipyrine/ferricyanide phenol detection described in the preceding section. However, it was found that product chloropyridinols absorb strongly in the near UV with λ_{max} of 325 nm at pH 7 (extinction coefficient ~8,400 $M^{-1}.cm^{-1}$). This was used to create a continuous microplate-based spectrophotometric assay. Assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 µM NH_4FeSO_4, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero and the increase in absorbance followed for 10 minutes at 325 nm in a microplate reader. The first 2 minutes of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 µl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9 µM chloropyridinol.

[00163] 5.3 – Colorimetric assay using 2-(2-chloro,4-nitrophenoxy)propionate

[00164] A convenient assay of AAD-12 was devised using 2-(2-chloro,4-nitrophenoxy)propionate (CNPP) as substrate. Cleavage of CNPP by AAD-12 releases 2-chloro,4-nitrophenol. This phenol has a bright yellow absorbance at 410 nm at pH 7 enabling the reaction to be followed continuously or by endpoint analysis. The presence of AAD-12

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activity can be monitored visually without the need for addition of further reagents. Microplate-based spectrophotometric assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate amount of CNPP (added from a 10 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of CNPP, enzyme, or α-ketoglutarate at time zero and the increase in absorbance followed for 10 min at 410 nm in a microplate reader. The first 2 min of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 μl final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1 μM 2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be $K_m = 31 \pm 5.5$ μM and $k_{cat} = 16.2 \pm 0.79$ min^{-1}.

Example 6 – In vitro activity of AAD-12 on various substrates

[00165] 6.1 – AAD-12 (v2) activity on (R,S)- dichlorprop, (R)-dichlorprop, (S)- dichlorprop and 2,4-D

[00166] Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4 μg purified AAD-12 (v2). (R,S)-dichlorprop (R,S-DCP) was tested at 1 mM and (R)-dichlorprop, (S)-dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in Figure 1, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4 μg AAD-12 (v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of α-ketoglutarate. After 5 minutes, the reaction was quenched, and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

[00167] AAD-12 (v2) has excellent activity on (R,S)-dichlorprop and (S)-dichlorprop and has minimal activity on (R)-dichlorprop. This indicates that AAD-12 (v2) has a clear (S)-enantiomeric preference. The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)-dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively.

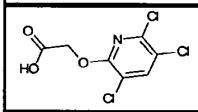
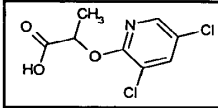
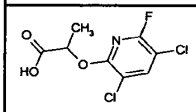
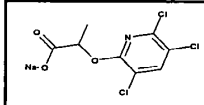
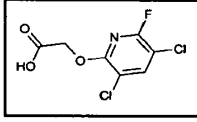
[00168] 6.2 – AAD-12 (v2) activity on pyridyloxyalkanoates

[00169] Using the pyridinol assay described in Example 5.2, five pyridyloxyalkanoates were assayed at 1 mM in a reaction mix containing 6.8 μg purified AAD-12 (v2). The rates of each

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reaction were monitored and are presented in **Table 8**. All five pyridyl-oxyalkanoates were cleaved to release pyridinols by AAD-12 (v2). The rates for the oxypropionate substrates 116844 and 91767 were somewhat faster than those for the corresponding acetates (triclopyr and 93833 respectively) indicating a preference of AAD-12 (v2) for oxypropionate over oxyacetate side chains. These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.

Table 8: Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8 µg AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of α-ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without α-ketoglutarate was subtracted from the rates with substrate

STRUCTURE	ID	Rate (mAU/min)	Rate relative to triclopyr
	triclopyr	97	1
	66357	225	2.3
	91767	190	0.8
	116844	257	1.4
	93833	118	0.5

[00170] 6.3 – Kinetic constants of AAD-12 (v2) for 2,4-D, (R,S)-DCP and triclopyr

[00171] The K_m and k_{cat} values of purified AAD-12 (v2) for the herbicides 2,4-D, (R,S)-dichlorprop and triclopyr were determined using the appropriate assay method. Substrate inhibition occurred at high concentrations (>1mM) of 2,4-D and (R,S)-DCP so concentrations below this were used to fit the data to the Michaelis-Menten equation using Grafit 4.0

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(Erithacus Software, UK). No substrate inhibition was noted for triclopyr up to 2 mM. The kinetic constants are summarized in **Table 9**. From these data, the rate of AAD-12 (v2) cleavage of triclopyr is ~5% that of 2,4-D, under maximal velocity conditions.

Table 9: Kinetic constants of AAD-12 (v2) for three herbicide substrates

Substrate	K_m, µM (±SE)	k_{cat}, min^{-1} (±SE)	Assay method	Substrate inhibition at 2 mM
2,4-D	102 (±18.4)	54.1 (±3.1)	Phenol detection	55%
(R,S)-dichlorprop	122 (±2.7)*	63.4 (±0.5)	Phenol detection	55%
Triclopyr	241 (±30)	2.6 (±0.1)	ΔA325 nm	0%

* Because of the (S)-enantiomeric preference of AAD-12, the K_m value was calculated assuming 50% of the racemic mixture was available as a substrate

Example 7 – Transformation into *Arabidopsis* and Selection

[00172] 7.1 – *Arabidopsis thaliana* growth conditions.

[00173] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00174] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00175] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 µmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

[00176] 7.2 – *Agrobacterium* transformation.

[00177] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

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[00178] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 µl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00179] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00180] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed Agrobacterium cells, suspended in 10 µl of water, at 100° C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

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[00181] 7.3 – *Arabidopsis* transformation.

[00182] *Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044μM benzylamino purine (10 μl/liter of 1 mg/ml stock in DMSO) and 300 μl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

[00183] 7.4 – Selection of transformed plants.

[00184] Freshly harvested T_1 seed [*AAD-12 (v1)* gene] was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00185] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00186] Seven days after planting (DAP) and again 11 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703

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L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-12 (v1)* (plant optimized gene) to provide phenoxy auxin herbicide resistance.

[00187] Random T_1 plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis* 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200g ae/ha). Tables 10 and 11 show comparisons drawn to an aryloxyalkanoate herbicide resistance gene (*AAD-1 v3*) previously described in PCT/US2005/014737.

[00188] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

[00189] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluroxypyr, could be effectively degraded *in planta*. Herbicides were applied to T_1 plants with use of a track sparyer in a 187 L/ha spray volume. T_1 plants that exhibited tolerance to 2,4-D DMA were further accessed in the T_2 generation.

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[00190] 7.5 – Results of selection of transformed plants.

[00191] The first *Arabidopsis* transformations were conducted using *AAD-12 (v1)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T_1 seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT + Liberty are used for selection. T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 10** compares the response of *AAD-12 (v1)* and control genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The *AAD-12 (v1)* gene imparted herbicide resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 10** simply to demonstrate the significant difference between the plants transformed with *AAD-12 (v1)* versus the wildtype or *PAT/Cry1F*-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or 6x field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

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Table 10. *AAD-12 v1* (plant optimized)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence compared to or *AAD-1 v3* (T_4) homozygous resistant population, PatCry1F transformed, auxin-sensitive control.

AAD-12 v1 gene T_1 transformants	% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control-buffer	6	0	0	0	0
50 g ae/ha 2,4-D	6	0	2	16	24
200 g ae/ha 2,4-D	6	1	1	11	18
800 g ae/ha 2,4-D	5	2	1	15	20
3200 g ae/ha 2,4-D	8	0	0	6	6
PAT/Cry1F (transformed control)	% Injury			% Injury Ave	Std Dev
Averages	<20%	20-40%	>40%		
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	4	1	5	31	16
200 g ae/ha 2,4-D	0	0	10	70	2
800 g ae/ha 2,4-D	0	0	10	81	8
3200 g ae/ha 2,4-D	0	0	10	91	2
Homozygous AAD-1 (v3) gene T_4 plants	% Injury			% Injury Ave	Std Dev
Averages	<20%	20-40%	>40%		
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	10	0	0	0	0
200 g ae/ha 2,4-D	10	0	0	0	0
800 g ae/ha 2,4-D	10	0	0	0	0
3200g ae/ha 2,4-D	9	1	0	2	6

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Table 11 shows a similarly conducted dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. The data shows that the herbicidally active (R-isomer of diclorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-diclorprop is one distinguishing characteristic that separates the two genes. (**Table 11**). AAD-1 and AAD-12 are considered R- and S-specific α-ketoglutarate dioxygenases, respectively.

Table 11. T_1 *Arabidopsis* response to a range of R-dichlorprop rates applied postemergence.

AAD-12 v1 gene	% Injury				% Injury	
Averages	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	6	0	0		0	0
50 g ae/ha R-dichlorprop	0	0	8		63	7
200 g ae/ha R-dichlorprop	0	0	8		85	10
800 g ae/ha R-dichlorprop	0	0	8		96	4
3200 g ae/ha R-dichlorprop	0	0	8		98	2
PAT/Cry1F		% Injury			% Injury	
Averages	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	10	0	0		0	0
50 g ae/ha R-dichlorprop	0	10	0		27	2
200 g ae/ha R-dichlorprop	0	0	10		69	3
800 g ae/ha R-dichlorprop	0	0	10		83	6
3200 g ae/ha R-dichlorprop	0	0	10		90	2
Homozygous AAD-1 (v3) gene		% Injury			% Injury	
T_4 plants	<20%	20-40%	>40%		Ave	Std Dev
Untreated control	10	0	0		0	0
50g ae/ha R-dichlorprop	10	0	0		0	0
200 g ae/ha R-dichlorprop	10	0	0		0	0
800 g ae/ha R-dichlorprop	10	0	0		0	0
3200 g ae/ha R-dichlorprop	10	0	0		0	0

[00192] 7.6 – *AAD-12 (v1)* as a selectable marker:

[00193] The ability to use *AAD-12 (v1)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. Approximately 50 T_4 generation Arabidopsis seed (homozygous for AAD-12 (v1)) was spiked into approximately 5,000 wildtype (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 follwed by 11 DAP. Since all individuals also contained the PAT gene comparison concomitant with the transformtation vetor, comparison of AAD-12 / 2,4-D selected and glufosinate selection was made.

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Treatments were applied with a DeVilbiss spray tip as previously describd. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate AAD-12 can effectively be used as an alternative selectable marker for a population.

[00194] 7.7 – Heritability.

[00195] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 55% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00196] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show *AAD12 (v1)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

[00197] 7.8 – Additional foliar applications herbicide resistance in AAD-12 *Arabidopsis*.

[00198] The ability of *AAD-12 (v1)* to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates. T_2 generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing *PAT* and the insect resistance gene *Cry1F* was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Dow AgroSciences) and 280-2240 g ae/ha fluroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ae/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

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[00199] These results (see **Table 12**) confirm that *AAD-12 (v1)* in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on transgenic non-AAD-12 control *Arabidopsis (Pat/Cry1F)*. This is the first report of an enzyme with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

Table 12. Comparison of T_2 AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

	Pyridyloxyacetic auxins	
	Ave % Injury 14DAT	
Herbicide Treatment	**Segregating T_2 *AAD-12 (v1)*plants (pDAB724.01.120)**	**Pat/Cry1f - Control**
280 g ae/ha Triclopyr	0	52
560 g ae/ha Triclopyr	3	58
1120 g ae/ha Triclopyr	0	75*
2240 g ae/ha Triclopyr	3	75*
280 g ae/ha Fluroxypyr	0	75*
560 g ae/ha Fluroxypyr	2	75*
1120 g ae/ha Fluroxypyr	3	75*
2240 g ae/ha Fluroxypyr	5	75*
	Inactive DCP metabolite	
280 g ae/ha 2,4-DCP	0	0
560 g ae/ha 2,4-DCP	0	0
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

* Plants in this experiment were stunted and severely epinastic, but remained green and did not receive injury ratings > 75%.

[00200] 7.9 – Molecular analysis of *AAD-12 (v1) Arabidopsis*.

[00201] Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-12 (v1)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-12 (v1)*. Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00202] Results showed all 2,4-D resistant plants assayed contained *PAT* (and thus by inference, *AAD-12 (v1)*). Copy number analysis showed total inserts ranged from 1 to 5

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copies. This correlates, too, with the *AAD-12 (v1)* protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

[00203] 7.10 – *Arabidopsis* transformed with molecular stack of *AAD-12 (v1)* and glyphosate resistance gene.

[00204] T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3759 plasmid (*AAD-12 (v1)* + *EPSPS*) coding for a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-12 (v1)* as the selectable marker as described in example 7.6. T_1 plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-12 (v1)* + *PAT* T_4 homozygous lines (pDAB724-transformed), and *PAT* + *Cry1F* homozygous line (transformed control). pDAB3759 and pDAB724 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

[00205] 7.11 – *AAD-12 Arabidopsis* genetically stacked with *AAD-1* to give wider spectrum of herbicide tolerance.

[00206] *AAD-12 (v1)* (pDAB724) and *AAD-1 (v3)* (pDAB721) plants were reciprocally crossed and F_1 seed was collected. Eight F_1 seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F_1 plants and subjected to Western analysis to confirm the presence of both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

[00207] One hundred F_2 seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F_2 plants survived glufosinate selection fitting an expected segregation ration for two independently assorting loci for glufosinate resistance (15 R : 1S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop + 560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are aconsistent with an expected segregation pattern (9R:

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6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlroprop or triclopyr). The results indicate that *AAD-12* (pDAB724) can be successfully stacked with *AAD-1* (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D + R-dichlorprop) and (2,4-D + fluroxypyr + triclopyr), respectively]. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F1 generation through paired sprays with herbicides that are exclusive between the AAD-1 and AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively, above).

Example 8 –WHISKERS-mediated transformation using imazethapyr selection

[00208] 8.1 – Cloning of *AAD-12 (v1)*.

[00209] The *AAD-12 (v1)* gene was cut out of the intermediate vector pDAB3283 as an NcoI/SacI fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1 : *AAD-12 (v1)* cassette. This construct was digested with NotI and PvuI to liberate the gene cassette and digest the unwanted backbone. This was ligated to NotI cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101, which contains ZmUbi1/*AAD-12 (v1)* / ZmPer5::OsAct1/AHAS /ZmLip.

[00210] 8.2 – Callus/suspension initiation.

[00211] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

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[00212] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00213] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

[00214] 8.3 – Cryopreservation and thawing of suspensions.

[00215] Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes

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within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

[00216] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

[00217] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00218] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After

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agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00219] Approximately 2mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

[00220] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 3 μM imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8) Plates were placed in boxes and cultured for an additional week.

[00221] Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C) containing 3 μM imazethapyr. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C in dark boxes.

[00222] 8.4- Protocol for Plant Regeneration. Putatively transformed isolates are typically first visible 5-8 weeks post-transformation. Any potential isolates are removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth is evident after approximately 2 weeks, an event is deemed to be resistant and is submitted for molecular analysis.

[00223] Regeneration is initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3 μM imazethapyr, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells are allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week, then higher light (40 $\mu Em^{-2}s^{-1}$) for another week, before being transferred to regeneration medium, 36 (3P), which is identical to 28 (3P) except that it lacks plant growth regulators. Small (3-5 cm) plantlets are removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once

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plantlets developed a sufficient root and shoot system, they are transplanted to soil in the greenhouse.

[00224] 8.5– Molecular Analysis: Maize Materials and Methods.

[00225] 8.5.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00226] 8.5.2 – Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl is placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well is overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

[00227] 8.5.3 – Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-12 (v1)* PTU are (Forward – ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:8) and (Reverse – CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR *AAD-12 (v1)* are (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:10) and (Reverse – CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute

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and 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00228] 8.5.4 – Southern blot analysis. Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μg of plasmid DNA is subjected to an overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85 % agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

Example 9. Protein detection from transformed plants via antibody

[00229] 9.1 – Extracting AAD-12 (v1) from plant leaves.

[00230] Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 μL plant extraction buffer (PBS containing 0.1 % Triton X-100 and 10 mM DTT). The tubes were shaken for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000x g. The supernatant containing the plant soluble proteins

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were analyzed for both total soluble protein (TSP) and AAD-12 (v1) using Western Blots and ELISA.

[00231] 9.2 – Bradford assay.

[00232] Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five microliters of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For BSA standards, concentrations were 2000, 1500, 1000, 750, 500, 250 and 125 μg/mL. The Protein Assay Concentrate (BioRad, Hercules, CA) was first diluted 5 fold in PBS and 250 μL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

[00233] 9.3 – Enzyme linked immuno-sorbent assay (ELISA).

[00234] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-12 antibody (0.5 μg/mL) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 μL standard AAD-12 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve, purified AAD-12 was diluted 2-fold serially from 250 to 3.9 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-12 antibody-HRP conjugate (0.5 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce, Rockford, IL), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 μL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-12 in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[00235] For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

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[00236] 9.4 – Western blotting analysis.

[00237] Plant extracts or AAD-12 standards (5 and 0.5 µg/mL) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-12 protein was detected by anti-AAD-12 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham, NJ).

Example 10 – Tobacco Transformation

[00238] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB3278, aka pDAS1580, *AAD-12 (v1) + PAT*), grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30°C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma).

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These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

[00239] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[00240] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-12 (v1)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00241] The DNA samples were diluted to 9 ng/µl and then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

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Table 13 Tobacco T0 events transformed with pDAS1580 (*AAD-12 (v1) + PAT*)

# Tube	Plant ID	Copy # PAT	PTU PCR AAD12	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance*
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+	*	*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable
24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High

* Discount of relative resistance made by tolerance to 560 g ae/ha fluroxypyr.

[00242] All events were also assayed for the presence of the *AAD-12 (v1)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR *AAD-12* were (SdpacodF ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:10) and (SdpacodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:11). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by

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72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably *AAD-12 (v1)* since these genes are physically linked) were regenerated and moved to the greenhouse.

[00243] 10.1 Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T_0 tobacco

[00244] T_0 plants from each of the 18 events were challenged with a wide range of 2,4-D, triclopyr, or fluroxypyr sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluroxypyr was likewise applied at 35, 140, or 560 g ae/ha. Triclopyr was appied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ae/ha (equivalent to 4X field rate). On the whole, *AAD-12 (v1)* events were more sensitive to fluroxypyr, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T0 plant responses to 560 g ae/ha fluroxypyr. Events were categorized into "low" (>40% injury 14 DAT), "medium" (20-40% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (**Table 13**).

[00245] 10.2 Verification of high 2,4-D tolerance in T_1 tobacco..

[00246] Two to four T_0 individuals surviving high rates of 2,4-D and fluroxypyr were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 7.4), followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (gene selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of 140 - 2240 g ae/ha 2,4-D dimethylamine salt (DMA), 70-1120 g

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ae/ha triclopyr or 35-560 g ae/ha fluroxypyr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity.

[00247] No injury was observed at 4X field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line but the greatest injury was observed with fluroxypyr, although this was short-lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT. (Table 14) It is important to note that untransformed tobacco is exceedingly sensitive to fluroxypyr. These results indicated commercial level 2,4-D tolerance can be provided by *AAD-12 (v1)*, even in a very auxin-sensitive dicot crop like tobacco and now across multiple species. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluroxypyr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

Table 14. Segregating *AAD-12* T_1 tobacco plants' response to phenoxy and pyridyloxy auxin herbicides.

Herbicide	KY160 – Wildtype	1580(1)-004 (high tolerance in T_0 generation)	1580(1)-018 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
140 g ae/ha 2,4-D DMA	45	0	0
560 g ae/ha 2,4-D DMA	60	0	0
2240 g ae/ha 2,4-D DMA	73	0	0
70 g ae/ha triclopyr	40	0	5
280 g ae/ha triclopyr	65	0	5
1120 g ae/ha triclopyr	80	0	8
35 g ae/ha fluroxypyr	85	0	8
140 g ae/ha fluroxypyr	93	0	10
560 g ae/ha fluroxypyr	100	3	18

[00248] 10.3 *AAD-12 (v1)* heritability in tobacco

[00249] A 100 plant progeny test was also conducted on seven T1 lines of *AAD-12 (v1)* lines. The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants were then sprayed with 560 g

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ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

10.4 – Field tolerance of pDAS1580 tobacco plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

[00250] Field level tolerance trials were conducted on three *AAD-12 (v1)* lines (events PDAS1580-[1]-018.001, PDAS1580-[1]-004.001 and PDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Mississippi. Tobacco transplants were grown in the greenhouse by planting T1 seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

[00251] The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after application.

[00252] *AAD-12 (v1)* event response to 2,4-D, triclopyr, and fluroxypyr are shown in **Table 15**. The non-transformed tobacco line was severely injured (63% at 14DAT) by 2,4-D at 560 g ae/ha which is considered the 1X rate. The *AAD-12 (v1)* lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8X rates, respectively. The non-transformed tobacco line was severely injured (53% at 14DAT) by the 2X rates of triclopyr (840 g ae/ha). *AAD-12 (v1)* lines demonstrated tolerance 2X rates of triclopyr with an average of 5% injury at 14DAT across the three lines. Fluroxypyr at 280 g ae/ha caused severe injury (99%) to the non-transformed line at 14DAA. *AAD-12 (v1)* lines demonstrated increased tolerance with an average of 11% injury at 14DAT.

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[00253] These results indicate that *AAD-12 (v1)* transformed event lines displayed a high level of resistance to 2,4-D, triclopyr and fluroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed tobacco.

Table 15. AAD-12 tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

Herbicide Treatment		Average % Injury across locations at 14 DAT			
Active Ingredient	Rate	Wild type	PDAS1580-[1]-004.001	PDAS1580-[1]-020.016	PDAS1580-[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxypyr	280 GM AE/HA	99	11	11	12

[00254] 10.5 Stacking of *AAD-12* to increase herbicide spectrum

[00255] *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) plants (*see* PCT/US2005/014737 for the latter) were reciprocally crossed and F_1 seed was collected. F_1 seed will be stratified and treated with 560 g ai/ha glufosinate. To the surviving plants, 560 g ae/ha dichlorprop (to confirm the presence of the *AAD-1 (v3)* gene) + 560 g ae/ha triclopyr dichlorprop (to confirm the presence of the *AAD-12 (v1)* gene) will be applied to the plants under the same spray regimen as used for the other testing. Plants shall be graded at 3 and 14 DAT. Tissue samples are taken from the F_1 plants and subjected to Western analysis to confirm the presence of both genes. The results will confirm that *AAD-12 (v1)* can be successfully stacked with *AAD-1 (v3)*, thus increasing the spectrum herbicides that may be applied to the crop of interest (phenoxyactetic acids + phenoxypropionic acids vs penoxyacetic acids + pyridyloxyacetic acids for AAD-1 and AAD-12, respectively). The complementary nature of herbicide cross resistance patterns allows convenient use of these two genes as conventionally stackable selectable markers.

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Example 11 - Soybean Transformation

[00256] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00257] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-12 (v1)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-12 (v1)*.

[00258] 11.1 Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00259] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly

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dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00260] 11.1.1 *Agrobacterium* preparation. The plasmid pDAB3759 contains the *AAD-12 (v1)* gene under the control of the *Casava vein mosaic virus* promoter. This plasmid also carries the *PAT* gene under the control of *Arabidopsis* Ubi10 promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. PDAB3759 also carries the glyphosate resistance trait described in section 2.4. This vector can be used in the transformation experiment described below. The construct pDAB3759 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

[00261] *Agrobacterium* cultures harboring pDAB3759 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

[00262] 11.1.2 – Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be

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rinsed five times with sterile water on hormone-free SHGA medium and allowed to germinate for 5 days at 24° C, with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.*, 1988).

[00263] Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 μM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

[00264] Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin , 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

[00265] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D

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selection pressure ranging from10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

[00266] The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

[00267] 11.1.3 – Progeny evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-12 (v1)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance will be assessed in the T_1 generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of *AAD-12 (v1)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

[00268] 11.2 Transformation Method 2: Aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00269] Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Patent No. 6,809,232 (Held *et al.*).

[00270] Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144, 96E692, or 180801500-17 were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3277 (see Table 7) using linearized DNA at a concentration of approximately 0.2 µg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos.

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With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing 1/2 B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

[00271] Variations of media referred to in **Table 16** were tested, *e.g.*, B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and 1/2 B5G which contained all ingredients of B5G medium at half strength.

[00272] Plants were selected after 5-8 months in selection and molecular analysis was carried out to check the integration of the transgene. Two T_0 events were generated and were positive by PCR for the presence of the AAD-12 gene. One plant was fertile and produced 12 normal T_1 seeds that will be tested for 2,4-D resistance as described in section 11.1.

Table 16. Growth Media for Soybean

	Ingredients in 1 Liter		
	B1-30	**B3**	**B5G**
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8g	8g	
Gelrite			2g
pH	5.8	5.8	5.8

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Example 12– *AAD-12 (v1)* in Cotton

[00273] 12.1 – Cotton transformation protocol.

[00274] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (**Table 17**) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00275] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (**Table 17**) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (**Table 17**) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (**Table 17**). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00276] *Agrobacterium* preparation. Inoculate 35ml of Y media (**Table 17**) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (**Table 17**) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

[00277] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison, MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.05mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for to the AAD-12 enzyme. Callus is then transferred to CG-media (**Table 17**), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (**Table 17**) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media,

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embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

[00278] Larger, well-developed embryos are isolated and transferred to DK media (**Table 17**) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

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Table 17. Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

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[00279] 12.2 – Experiment specifics.

[00280] For this experiment 500 cotyledon segments were treated with pDAB724. The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage; then callus lines that are embryogenic will be sent for Western analysis.

[00281] 12.3 – Plant regeneration.

[00282] *AAD-12 (v1)* cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the *AAD-12 (v1)* gene provides resistance to 2,4-D in cotton, both the *AAD-12 (v1)* cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187 L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T_1 seed or outcrossed with an elite cotton line to produce F_1 seed. The subsequent seed produced will be planted and evaluated for herbicide resistance as previously described.

Example 13 – *Agrobacterium* Transformation of Other Crops

[00283] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g.,* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.*, 1997.

[00284] The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *AAD-12 (v1)*, for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato

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(*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa*, *perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans (*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, and other families), and Clover (*Leguminosae*). Such plants, with *AAD-12 (v1)* genes, for example, are included in the subject invention.

[00285] *AAD-12 (v1)* has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluroxypyr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited to: alder, ash, aspen, beech, birch, cherry, eucalyptys, hickory, maple, oak, pine, and poplar. Use of auxin resistance for the selective control in ornamental species is also within the scope of this invention. Examples could include, but not be limited to, roses, Euonymus, petunia, begonia, and marigolds.

Example 14 – Further Evidence of Surprising Results: *AAD-12 vs. AAD-2*

[00286] 14.1 – *AAD-2 (v1)* initial cloning.

[00287] Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

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[00288] The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

[00289] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H_2O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00290] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO:6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:7) primers, per manufacturers

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instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[00291] 14.2 – Completion of *AAD-2 (v1)* binary vector.

[00292] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. *See* PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:12) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:13) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI – NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

[00293] 14.3 – Evaluation of transformed *Arabidopsis*.

[00294] Freshly harvested T_1 seed transformed with a plant optimized *AAD-12 (v1)* or native *AAD-2 (v1)* gene were planted and selected for resistance to glufosinate as previously described Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

[00295] *AAD-12 (v1)* and *AAD-2 (v1)* did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants.

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Surprisingly, *AAD-2 (v1)*and *AAD-2 (v2)* transformants were far less resistant to 2,4-D than *AAD-12 (v1)* genes.both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (*see* PCT/US2005/014737). Conversely, AAD-12 had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D (Table 10). Tolerance improved slightly for plant-optimized *AAD2 (v2)* versus the native gene; however, comparison of both *AAD-12* and *-2* plant optimized genes indicates a significant advantage for *AAD-12 (v1) in planta.*

[00296] These results are unexpected given that *in vitro* comparison native microbial genes of *AAD-2 (v1)* (*see* PCT/US2005/014737) and *AAD-12 (v2)* indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. No large difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes (*see* PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of AAD-12 (v1) *in planta* for imparting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides is unexpected.

Example 15 – Preplant Burndown Applications

[00297] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

[00298] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and

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activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (*see* federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With *AAD-12 (v1)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. Likewise, the unique attributes of AAD-12 allowing degradation of triclopyr and fluroxypyr increase utility by allowing substitution or tank mixesof 70-1120 or 35-560 g ae/ha of triclopyr andfluroxypyr, respectively, to increase spectram and/or increase the abilty to control perennial or viney weed species.

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Example 16 – In-Crop Use of Phenoxy Auxins Herbicides in Soybeans, Cotton, and Other Dicot Crops Transformed Only with *AAD-12 (v1)*

[00299] *AAD-12 (v1)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D and MCPA) and pyridyloxy auxins (trickopyr and fluroxypyr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00300] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluroxypyr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

[00301] Further benefits could include tolerance to 2,4-D, triclopyr or fluroxypyr required before planting following aryloxyacetic acid auxin herbicide application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D, triclopyr or fluroxypyr. Dicamba (and other herbicides) can still be used for the subsequent control of *AAD-12 (v1)*-transformed dicot crop volunteers.

[00302] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by *AAD-12 (v1)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

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Example 17 - In-Crop Use of Phenoxy Auxin and Pyridyl Auxin Herbicides in *AAD-12 (v1)* Only Transformed Corn, Rice, and Other Monocot Species

[00303] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-12 (v1)* would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D,.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. *AAD-12 (v1)*-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00304] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, or fluroxypyr. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-12 (v1)* HTC combination strategy, whether one rotates crops species or not. The increased tolerance of corn, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 18 – *AAD-12 (v1)* Stacked With Glyphosate Tolerance Trait in Any Crop

[00305] The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional

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GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-12 (v1)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-12 (v1)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more

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preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Tricopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimin. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D, triclopyr, or fluroxypyr.

[00306] One skilled in the art of weed control will recognize that use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 19 - *AAD-12 (v1)* Stacked with Glufosinate Tolerance Trait in Any Crop

[00307] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

[00308] By stacking *AAD-12 (v1)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy,

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flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

   i) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species. Triclopyr and fluroxypyr would be acceptable components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) A multiple combination of glufosinate (200-500 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00309] One skilled in the art of weed control will recognize that use of one or more commercial aryloxyacetic auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each

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alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - *AAD-12 (v1)* Stacked with *AHAS* Trait in Any Crop

[00310] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* Commercial imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking *AAD-12 (v1)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monoct crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and these auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. For triclopyr, application rates would

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typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp.* can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. See rates above for triclopyr or fluroxypyr. iii)

b) A mulitple combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00311] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by *AAD-12 (v1)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-12 (v1)* in Rice

[00312] 21.1 – Media description.

[00313] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

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[00314] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3–4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (1/2MSH50).

[00315] 21.2 – Tissue culture development.

[00316] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants

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regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week prior to storage at 4° C.

[00317] 21.3 – Microprojectile bombardment.

[00318] All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB4101, 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[00319] 21.4 – Tolerance testing.

[00320] Rice plantlets at the 3-5 leaf stage will be sprayed with a selective dose (~70-140 g ae/ha) of imazethapyr + methylated seed oil (1%) by track sprayer at 187 L/ha spray volume. Rating for sensitivity or resistance will be done at 10-14 days after treatment (DAT).

[00321] 21.5 – Tissue harvesting, DNA isolation and quantification.

[00322] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

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Example 22 – *AAD-12 (v1)* in Canola

[00323] 22.1 – Canola transformation.

[00324] The *AAD-12 (v1)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained *AAD-12 (v1)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi10 promoter (*see* section 2.4).

[00325] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00326] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00327] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00328] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 2-3 weeks.

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[00329] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/l MES, 5 or 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00330] The transformed T_0 plants will be self-pollinated in the greenhouse to obtain T_1 seed. The T_0 plants and T_1 progeny will be sprayed with a range of herbicide (2,4-D; triclopyr; fluroxypyr; glyphosate; and glufosinate) rates to establish the level of protection by the *AAD-12 (v1)* gene as previously described above.

[00331] 22.2 – "Molecular analysis": canola materials and methods

[00332] 22.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ul.

[00333] 22.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-12 (v1)* were (SEQ ID NO:10) (forward) and (SEQ ID NO:11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-12 (v1)* events tested positive. Three negative control samples tested negative.

[00334] 22.2.3 – ELISA.

[00335] Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have

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minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 18.

Table 18 Expression of AAD-12 in Canola plants

Sample #	[TSP] (µg/mL)	[AAD-12] (ng/mL)	Expression (ppm TSP) (ELISA)	Western
31	5614.96	1692.12	301.36	++++
33	4988.26	2121.52	425.30	++++
38	5372.25	3879.09	722.06	++++
39	2812.77	41.36	14.71	+
40	3691.48	468.74	126.98	+++
Control 1	2736.24	0.00	0.00	-
Control 2	2176.06	0.00	0.00	-
Control 3	3403.26	0.00	0.00	-

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Claims

1. A transgenic plant comprising a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide.

2. The transgenic plant of claim 1, said polynucleotide also conferring resistance to a phenoxy auxin herbicide.

3. A method of controlling at least one weed in a field, said method comprising growing at least one transgenic plant of claim 1 in said field, and applying a pyridyloxy auxin herbicide to at least a portion of said field.

4. A method of controlling at least one weed in a field, said method comprising growing at least one plant of claim 2 in said field, and applying a phenoxy auxin herbicide to at least a portion of said field.

5. The method of claim 3, further comprising applying a phenoxy auxin herbicide to at least a portion of said field.

6. The method of claim 3, said pyridyloxy auxin herbicide selected from the group consisting of fluroxypyr and triclopyr.

7. The method of claim 4, said phenoxy auxin herbicide selected from the group consisting of 2,4-D and MCPA.

8. The plant of claim 1, said plant further comprising at least one additional herbicide resistance gene.

9. The plant of claim 8, said at least one additional herbicide resistance gene rendering said plant resistant to at least one herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

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10. The plant of claim 1 wherein said plant is selected from the group consisting of soybean, canola, tobacco, Arabidopsis, corn, cotton, and rice.

11. The plant of claim 1 wherein said polynucleotide encodes an AAD-12 protein having aryloxyalkanoate dioxygenase activity, said AAD-12 protein having at least 95% identity to a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

12. The plant of claim 1 wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

13. A polynucleotide comprising a codon composition that is optimized for expression in an organism other than *Delftia acidovoransa*, wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity.

14. The polynucleotide of claim 13 wherein said polynucleotide comprises SEQ ID NO:5.

15. The polynucleotide of claim 13 wherein said codon composition that is optimized for expression in a plant.

16. The polynucleotide of claim 15 wherein said polynucleotide comprises SEQ ID NO:3.

17. A plant cell comprising a heterologous polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

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18. A method of selecting for a transformed plant cell of claim 17 wherein said method comprises subjecting a plurality of plant cells to transformation with said polynucleotide, then growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin herbicide.

19. The method of claim 18 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

20. A seed of a plant of claim 1.

21. A plant grown from the seed of claim 20.

22. A part, progeny, or asexual propagate of the plant of claim 1.

23. The method of claim 4 wherein said method is used to treat and/or prevent herbicide-resistant weeds, and/or to treat weeds that are naturally tolerant to control by other herbicides.

24. The method of claim 5 wherein said method further comprises applying a third herbicide.

25. The method of claim 24 wherein said phenoxy auxin herbicide is 2,4-D; said pyridyloxy auxin herbicide is selected from the group consisting of triclopyr and fluroxypyr; and said third herbicide is selected from the group consisting of glyphosate, glufosinate, paraquat, chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron, cloransulam-methyl, diclosulam, dicamba, florasulam, flumetsulam, metosulam, penoxsulam imazamox, imazethapyr, imazaquin, dicamba, mesotrione, isoxaflutole, sulcotrione, flufenpyr, pyraflufen, butafenacil, amidosulfuron, bensulfuron, chlorimuron, cinosulfuron, flupyrsulfuron, foramsulfuron, primisulfuron, prosulfuron,

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rimsulfuron, thifensulfuron, triasulfuron, triflusulfuron, flumiclorac, flumioxazin, fluthiacet, carfentrazone, sulfentrazone, acifluorfen, fomesafen, lactofen, and oxyfluorfen.

26. A method of controlling at least one weed in a field, said method comprising planting in said field seed of at least one transgenic plant, said plant comprising

a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide, and

a second heterologous polynucleotide encodes an enzyme conferring resistance to at least one other herbicide;

said method further comprising applying to at least a portion of said field a first herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide; and applying said at least one other herbicide to said at least a portion of said field.

27. The method of claim 26 wherein said herbicides are applied sequentially or concurrently.

28. The method of claim 26 wherein said first herbicide is a phenoxy auxin herbicide.

29. The method of claim 26 wherein said first herbicide is selected from the group consisting of 2,4-D, triclopyr, and fluroxypyr.

30. The method of claim 26 wherein said at least one other herbicide is selected from the group consisting of glyphosate, glufosinate, paraquat, chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron, cloransulam-methyl, diclosulam, dicamba florasulam, flumetsulam, metosulam, penoxsulam imazamox, imazethapyr, imazaquin, dicamba, mesotrione, isoxaflutole, sulcotrione, flufenpyr, pyraflufen, butafenacil, amidosulfuron, bensulfuron, chlorimuron, cinosulfuron, flupyrsulfuron, foramsulfuron, primisulfuron, prosulfuron, rimsulfuron, thifensulfuron, triasulfuron, triflusulfuron, flumiclorac, flumioxazin, fluthiacet, carfentrazone, sulfentrazone, acifluorfen, fomesafen, lactofen, and oxyfluorfen.

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31. A method of assaying a protein for its ability to cleave a pyridyloxyalkanoate herbicide, said method comprising providing a sample comprising said protein and a pyridyloxy auxin herbicide, and assaying said sample for presence of a chloropyridinol by ultraviolet absorption.

32. A method of assaying a protein for aryloxyalkanoate dioxygenase activity wherein said method comprises providing a sample comprising said protein and 2-(2-chloro,4-nitrophenoxy) propionate and/or acetate, and colorimetrically assaying said sample for presence of a 2-chloro,4-nitrophenol.

33. An expression vector comprising a nucleic acid molecule encoding a protein that enzymatically degrades at least one herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein said nucleic acid molecule hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

34. The expression vector of claim 33 wherein said promoter is a plant promoter.

35. The expression vector of claim 33 comprising a promoter selected from a cassava vein mosaic virus promoter, CaMV 35S promoter, Figwort Mosaic Virus promoter, rice actin promoter, phaseolin promoter, *Arabidopsis thaliana* Ubiquitin 10 promoter, maize ubiquitin promoter, *Arabidopsis thaliana* Act2 promoter, *Arabidopsis thaliana* Ubiquitin 11 promoter, and *Arabidopsis thaliana* Ubiquitin 3 promoter.

36. The method of claim 4 wherein said plant is resistant to a herbicide formulation comprising glyphosate.

37. A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said first herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin.

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38. The method of claim 37 wherein said first herbicide is an acid, an inorganic salt, an organic salt, or an ester.

39. The method of claim 37 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

40. The method of claim 39 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate.

41. A method of detecting whether a plant comprises a polynucleotide of claim 15 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

42. The method of claim 41 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

43. The method of 41 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

44. The method of 42 wherein said method comprises using an antibody to detect for the presence of said protein.

45. The plant of claim 1 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

46. The plant of claim 1 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

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47. The method of claim 4 wherein said plant is resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, imazethapyr, chlorsulfuron, dicamba, mesotrione, isoxaflutole, and butafenacil.

48. The method of claim 4 wherein said herbicide is an achiral phenoxy auxin.

49. The method of claim 26 wherein said plant is a monocot.

50. The method of claim 49 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, warm and cool-season turf grass, oats, sorghum, and pasture grasses.

51. The method of claim 26 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

52. The method of claim 51 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

53. A method of conferring herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a polynucleotide encoding an enzyme capable of using a pyridyloxy auxin herbicide and a phenoxy auxin herbicide as a substrate.

54. A method of controlling glyphosate-resistant, naturally glyphosate tolerant, and/or naturally glufosinate tolerant weeds in a field of glyphosate and/or glufosinate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 13, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

55. The method of claim 54 wherein said herbicide is a phenoxy auxin.

56. The method of claim 55 wherein said phenoxy auxin is 2,4-D.

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57. A method of controlling weeds that are resistant to an ALS-inhibiting herbicide in a field of crop plants that comprise a polynucleotide of claim 13, said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

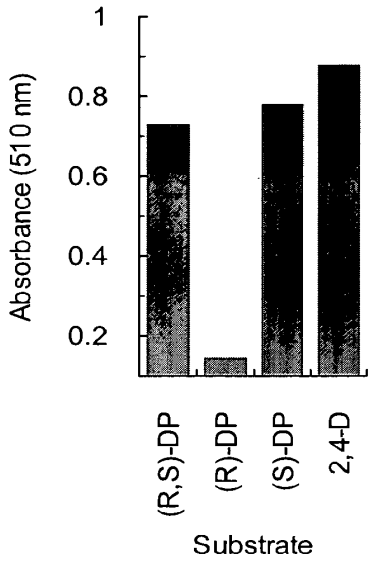
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Abstract

[00336] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide tolerant crop trait and selectable marker opportunities.

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Figure 1. Activity of AAD-12(v2) on 2,4-D and enantiomers of dichlorprop. 4.4 µg AAD-12(v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of α-ketoglutarate. After 5 min the reaction was quenched and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.



J:\DAS\DAS-129P\Figure 1.doc

Sequences

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tacatcggct acggcatgga caccaccgcc acgcccctgc gcccgctggt caaggtgcat 600

cccgagaccg gccgcccctc gctgctgatc ggccgccatg cccatgccat cccgggcatg 660

gacgccgccg aatccgagcg cttcctggaa ggcctggtcg actgggcctg ccaggcgccg 720

cgggtgcatg cccaccaatg ggccgccggc gacgtggtgg tgtgggacaa ccgctgcctg 780

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1 5 10 15

Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Phe Ala
20 25 30

Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro Gly
35 40 45

Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe Gly
50 55 60

Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn Val
65 70 75 80

Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp Asp
85 90 95

Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser Thr
100 105 110

Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val Val
115 120 125

Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala Tyr
130 135 140

Asp Ala Leu Asp Glu Ala Thr Arg Ala Leu Val His Gln Arg Ser Ala
145 150 155 160

Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln Gln
165 170 175

```
Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr Pro
            180                 185                 190

Leu Arg Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser Leu
        195                 200                 205

Leu Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala Glu
    210                 215                 220

Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala Pro
225                 230                 235                 240

Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp Asp
                245                 250                 255

Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe Lys Leu Pro
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Arg Val Met Trp His Ser Arg Leu Ala Gly Arg Pro Glu Thr Glu Gly
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Ala Ala Leu Val
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caacatgcac tcttgatctt ccctgggcaa cacctcagca atgaccaaca gattaccttt    180

gctaaacgct ttggagcaat tgagaggatt ggcggaggtg acattgttgc catatccaat    240

gtcaaggcag atggcacagt gcgccagcac tctcctgctg agtgggatga catgatgaag    300

gtcattgtgg gcaacatggc ctggcacgcc gactcaacct acatgccagt catggctcaa    360

ggagctgtgt tcagcgcaga agttgtccca gcagttgggg gcagaacctg ctttgctgac    420

atgagggcag cctacgatgc ccttgatgag gcaacccgtg ctcttgttca ccaaaggtct    480

gctcgtcact cccttgtgta ttctcagagc aagttgggac atgtccaaca ggccgggtca    540

gcctacatag gttatggcat ggacaccact gcaactcctc tcagaccatt ggtcaaggtg    600
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catcctgaga ctggaaggcc cagcctcttg atcggccgcc atgcccatgc catccctggc    660

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cccagagtcc atgctcacca atgggctgct ggagatgtgg ttgtgtggga caaccgctgt    780

ttgctccacc gtgctgagcc ctgggatttc aagttgccac gtgtgatgtg gcactccaga    840

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Ala Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro
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Gly Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe
    50                  55                  60

Gly Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn
65                  70                  75                  80

Val Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp
                85                  90                  95

Asp Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser
            100                 105                 110

Thr Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val
        115                 120                 125

Val Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala
    130                 135                 140

Tyr Asp Ala Leu Asp Glu Ala Thr Arg Ala Leu Val His Gln Arg Ser
145                 150                 155                 160
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Ala Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln
                165                 170                 175

Gln Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr
            180                 185                 190

Pro Leu Arg Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser
        195                 200                 205

Leu Leu Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala
    210                 215                 220

Glu Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala
225                 230                 235                 240

Pro Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp
                245                 250                 255

Asp Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe Lys Leu
            260                 265                 270

Pro Arg Val Met Trp His Ser Arg Leu Ala Gly Arg Pro Glu Thr Glu
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Gly Ala Ala Leu Val
    290
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caacatgctc tcctgatttt cccaggtcag cacctgtcca acgaccagca aatcactttt 180

gcaaaacgct tcggtgcgat cgaacgtatc ggtggcggtg atattgtggc gatctccaac 240

gtaaaagcgg atggtactgt acgtcagcac agcccggcgg agtgggacga tatgatgaag 300

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gcgcgtcact ctctggtgta ttcccagagc aaactgggcc acgttcagca agcgggctcc 540

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ccgcgtgtgc atgctcacca gtgggcagct ggcgacgtgg ttgtatggga taaccgctgc 780

ctgcttcacc gtgcagaacc gtgggacttt aagctgccac gtgttatgtg gcacagccgt 840

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<400> 15
gagctcctat cactccgccg cctgctgctg cac 33

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
12/091,896	Terry R. Wright	DAS-129XC1

23557
SALIWANCHIK LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
PO BOX 142950
GAINESVILLE, FL 32614-2950

INTERNATIONAL APPLICATION NO.	
PCT/US2006/042133	
I.A. FILING DATE	PRIORITY DATE
10/27/2006	10/28/2005

CONFIRMATION NO. 6428
371 FORMALITIES LETTER

OC000000031355282

Date Mailed: 08/04/2008

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as a Designated Office (37 CFR 1.494):

- Priority Document
- Copy of the International Application filed on 04/28/2008
- Biochemical Sequence Diskette filed on 04/28/2008
- Biochemical Sequence Listing filed on 04/28/2008
- U.S. Basic National Fees filed on 04/28/2008
- Priority Documents filed on 04/28/2008
- Specification filed on 04/28/2008
- Claims filed on 04/28/2008
- Abstracts filed on 04/28/2008
- Drawings filed on 04/28/2008
- Paper nucleotide sequence listings filed on 04/28/2008

The applicant needs to satisfy supplemental fees problems indicated below.

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.
- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.492(h) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**130** for a Large Entity:

- $**130** Surcharge.

Additionally the following defects have been observed:

- The International Search Report has not be supplied, the Search Fee has been adjusted to reflect this omission.

FORM PCT/DO/EO/905 (371 Formalities Notice)

Corteva Exhibit 2014 - Page 326 of 1122
Inari v. Corteva - PGR2023-00022

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 32 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

CHARITTA A SHELTON

Telephone: (703) 308-9140 EXT 207

FORM PCT/DO/EO/905 (371 Formalities Notice)

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 08/04/2008

CBURT1 SALE #00000015 Mailroom Dt: 04/28/2008 190065 12091896
01 FC : 1632 510.00 DA

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 08/04/2008

CBURT1 ADJ #00000015 Mailroom Dt: 04/28/2008
Seq No: 3163 Sales Acctg Dt: 04/29/2008 190065 12091896
02 FC : 1641 100.00 CR

UNITED STATES NATIONAL STAGE WORKSHEET (DO/EO)

CHARITTA SHELTON, PAS

U. S. Application No. 12/091896

Publication Date 5/10/2007 **WIPO number** 2007/053487

International App. # PCT/ US2006/042133 **Date** 10/27/2006

Publication language: English Priority doc. date 10/28/2005

Total Claims 15 **Chargeable claims** 15 **Independent claims** 2 **multiple claims** ___

371 Basic filing fee: ___ ✓ **insufficient** ___ SME fee paid ___ **Search fee US** ___ **IPER meets Art.33 (1)-(4) no fees** ___

Original claims replaced by Art.19 and/or Art.34 amendment ___

Total drawing sheets 3 foreign text not translated ___

Oath/Declaration: Unsigned ___ **Defective** ___ **Completion date** ___

Biochemical Seq. Diskette ___ error report date ___ entered date ___

Biochemical Seq. listing ___ CRF statement in file ___

Copy of ISR 210/220 N/A references ___ Copy of ISA 237 N/A 373 ___ IPER 409 ___

Art.34 annexes ___ not entered (see notes) ___ Art.19 Amendment ___

Preliminary Amendment ___ ✓ used to calculate claim fee: yes ___ no ___ / 2nd Amendment dated ___

Information Disclosure Statement ___ / filed after original date ___ / 2nd IDS dated ___

Request for Immediate Examination: yes ___ **no** ✓

Substitute Specification ___ Assignment filed ___

Priority Doc. Present: ___

Power of Attorney ___ doc. dated ___

PCT/RO/101___ U.S. Only: yes __No _X_

Early publication request ADS ___ Petition to revive ___ Petition 1.47 ___

IB 306 filed for changes: ___ Other IB papers ___

---

35 USC Receipts of Request 4-28-08

Completion USC 371 Requirements ___

Notice of Missing Requirements (905) 8.2.2008

Notice of Acceptance (903) ___

Notice of regarding CRF Disk (922) ___

Notice of insufficient fees (923) ___

Notice of Defective Response (916) ___

Notice of Abandonment (909) ___

--- **Notes:** ----------

PATENT APPLICATION FEE DETERMINATION RECORD
Effective September 30, 2007

Application or Docket Number: 12109/896

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)	SMALL ENTITY TYPE ☐ RATE	FEE	OR	OTHER THAN SMALL ENTITY RATE	FEE
U.S. NATIONAL STAGE FEES							
BASIC FEE	SMALL ENT. = $ 150	LARGE ENT. = $ 300	BASIC FEE	$155	OR	BASIC FEE	$310
EXAMINATION FEE	Satisfies PCT Article 33(1)-(4) = $ 50 / $ 100	All other situations = $ 100 / $ 200	EXAM. FEE	$105		EXAM. FEE	$210
SEARCH FEE	U.S. is ISA = $ 50 / $ 100 ALL other countries = $ 200 / $ 400	ALL other situations = $ 250 / $ 500	SEARCH FEE	$205		SEARCH FEE	$510
FEE FOR EXTRA SPEC. PGS.	minus 100 =	/ 50 =	X $ 130 =			X $ 260 =	
TOTAL CHARGEABLE CLAIMS	15 minus 20 =	*	X $ 25 =		OR	X $ 50 =	
INDEPENDENT CLAIMS	2 minus 3 =	*	X $ 105 =		OR	X $ 210 =	
MULTIPLE DEPENDENT CLAIM PRESENT			+ $ 185 =		OR	+ $ 370 =	
			TOTAL		OR	TOTAL	1030

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
AMENDMENT A	Total	*	Minus	**	=
	Independent	*	Minus	***	=
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐				

SMALL ENTITY RATE	ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	ADDITIONAL FEE
X $ 25 =		OR	X $ 50 =	
X $ 100 =		OR	X $ 200 =	
+ $ 180 =		OR	+ $ 360 =	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
AMENDMENT B	Total	*	Minus	**	=
	Independent	*	Minus	***	=
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐				

RATE	ADDITIONAL FEE	OR	RATE	ADDITIONAL FEE
X $ 25 =		OR	X $ 50 =	
X $ 100 =		OR	X $ 200 =	
+ $ 180 =		OR	+ $ 360 =	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '20', enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '3', enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET (FOR USE WITH FORM PTO-875)	SERIAL NO.	FILING DATE
	12/091896	

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
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TOTAL IND.						
TOTAL DEP.						
TOTAL CLAIMS						

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
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	88					

MULTIPLE DEPENDENT CLAIM CALCULATION SHEET (FOR USE WITH FORM PTO-875)	FEE	SERIAL NO. 12/091896	FILING DATE

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMEN			AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMEN	
	IND.	DEP.	IND.	DEP.	IND.	DEP.		IND.	DEP.	IND.	DEP.	IND.	DEP.
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TOTAL IND.		↔	2	⬇		⬇	TOTAL IND.		⬇		⬇		⬇
TOTAL DEP.		⬅	13	⬅		⬅	TOTAL DEP.		⬅		⬅		⬅
TOTAL CLAIMS			15				TOTAL CLAIMS						

PTO - 1360 (REV. 11/04)

U.S. DEPARTMENT of COMMERCE
Patent and Trademark Office

PATENT COOPERATION TREATY

RECEIVED

MAR 3 1 2008

From the INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing (*day/month/year*)
Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US06/42133	International filing date (*day/month/year*) 27 October 2006 (27.10.2006)
Applicant DOW AGROSCIENCES LLC	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70.

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 *bis*.1 and 90 *bis*.3, respectively, before the completion of the technical preparations for international publication.

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/ US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Vinod Kumar Telephone No. (571) 272-4445

Form PCT/ISA/220 (April 2002) *(See notes on accompanying sheet)*

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing (*day/month/year*) 28 MAR 2008
Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US06/42133	International filing date (*day/month/year*) 27 October 2006 (27.10.2006)
Applicant DOW AGROSCIENCES LLC	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70.

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 *bis*.1 and 90 *bis*.3, respectively, before the completion of the technical preparations for international publication.

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/ US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Vinod Kumar Telephone No. (571) 272-4445

Form PCT/ISA/220 (April 2002) *(See notes on accompanying sheet)*

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) the claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers, claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

If a demand for international preliminary examination is made, the written opinion of the International Searching Authority will, except in certain cases where the International Preliminary Examining Authority did not act as International Searching Authority and where it has notified the International Bureau under Rule 66.1*bis*(b), be considered to be a written opinion of the International Preliminary Examining Authority. If a demand is made, the applicant may submit to the International Preliminary Examining Authority a reply to the written opinion together, where appropriate, with amendments before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later (Rule 43*bis*.1(c)).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see the *PCT Applicant's Guide*, Volume II.

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NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*, a publication of WIPO.

In these Notes, "Article," "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Volume I/A, Annexes B1 and B2).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, Volume I/A, paragraph 296).

What parts of the international application may be amended ?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Preliminary Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When ? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How ? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference DAS-129XCI	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US06/42133	International filing date *(day/month/year)* 27 October 2006 (27.10.2006)	(Earliest) Priority Date *(day/month/year)* 28 October 2005 (28.10.2005)
Applicant DOW AGROSCIENCES LLC		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the Report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   ☒ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   ☒ contained in the international application in written form.
   ☒ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. ☐ **Certain claims were found unsearchable** (See Box I).
3. ☒ **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____
   ☐ as suggested by the applicant. ☒ None of the figures
   ☐ because the applicant failed to suggest a figure.
   ☐ because this figure better characterizes the invention.

Form PCT/ISA/210 (first sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/42133

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-84 (SEQ ID NOs: 1 and 2)

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/42133

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8): **A01H 5/00**(2006.01);**C12N 15/82**(2006.01);**C12N 5/04**(2006.01)

USPC: 800/295;800/300;800/301;800/302;800/278;800/289;435/468;435/419
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 800/295; 800/300; 8000/301; 8000/302; 8000/278; 8000/289; 435/468; 435/419

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Kaphammer et al. (US Patent No. 5,608,147, Issued March 4, 1997).	15-21, 30-32, 73-76
---		---
Y		42-43, 77
Y	Perlak et al. (PNAS, 88:3324-3328, 1991).	42
Y	Kasuga et al. (Nature Biotechnology, 17:287-291, 1999).	43
Y	Verdaguer et al. (Plant Molecular Biology, 31:1129-1139, 1996).	77

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
28 November 2007 (28.11.2007)

Date of mailing of the international search report
28 MAR 2008

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner of Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (571) 273-3201

Authorized officer
Vinod Kumar
Telephone No. (571) 272-4445

Form PCT/ISA/210 (second sheet) (July 1998)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-84 drawn to a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 1, expression cassette, transgenic plant cell, or transgenic plant comprising said polynucleotide.

Group II, claim(s) 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-84 drawn to a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 3, expression cassette, transgenic plant cell, or transgenic plant comprising said polynucleotide.

Group III, claim(s) 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81 and 83-84 drawn to a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 5, expression cassette, transgenic plant cell, or transgenic plant comprising said polynucleotide.

Group IV, claim(s) 5-7, 22-23, 66-67, and 78 drawn to a method of selecting for transgenic plant cell comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 1.

Group V, claim(s) 5-7, 22-23, 66-67, and 78 drawn to a method of selecting for transgenic plant cell comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 3.

Group VI, claim(s) 5-7, 22-23, 66-67, and 78 drawn to a method of selecting for transgenic plant cell comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 5.

Group VII, claim(s) 8-14, 24-27, 33-37, 44-59, 65, 68-72, 82, and 85-88 drawn to a method of controlling weeds or protecting crops comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 1.

Group VIII, claim(s) 8-14, 24-27, 33-37, 44-59, 65, 68-72, 82, and 85-38 drawn to a method of controlling weeds or protecting crops comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 3.

Group IX, claim(s) 8-14, 24-27, 33-37, 44-59, 65, 68-72, 82, and 85-88 drawn to a method of controlling weeds or protecting crops comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 5.

Group X, claim(s) 28-29, 39, and 41 drawn to a method of assaying a protein, or wherein said protein is encoded by SEQ ID NO: 1.

Group XI, claim(s) 28-29, 39, and 41 drawn to a method of assaying a protein, or wherein said protein is encoded by SEQ ID NO: 3.

Group XII, claim(s) 28-29, 39, and 41 drawn to a method of assaying a protein, or wherein said protein is encoded by SEQ ID NO: 5.

Group XIII, claim(s) 38, and 40 drawn to a method of detecting a polynucleotide, or wherein said polynucleotide is defined in SEQ ID NO: 1.

Group XIV, claim(s) 38, and 40 drawn to a method of detecting a polynucleotide, or wherein said polynucleotide is defined in SEQ ID NO: 3.

Group XV, claim(s) 38, and 40 drawn to a method of detecting a polynucleotide, or wherein said polynucleotide is defined in SEQ ID NO: 5.

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INTERNATIONAL SEARCH REPORT

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The inventions listed as Groups I-XV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking Groups I-XV appear to be a polynucleotide encoding a protein having aryloxyalkanoate dioxygenase activity. However, Schleinitz et al. (NCBI, GenBank Sequence Accession No. AY327575, Published September 8, 2004) teach a polynucleotide sequence encoding2, 4-dichlorophenoxypropionate cleavage. Therefore, the technical feature linking the inventions of Groups I-XV does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

Applicants are reminded that different nucleotide sequences and amino acid sequences are structurally distinct chemical compounds and are unrelated to one another. These sequences are thus deemed to normally constitute different inventive concepts.

Applicants are reminded that claim 27 is directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid.

Continuation of B. FIELDS SEARCHED Item 3:
WEST (USPT, PGPB), Agricola, CAplus, Biosis, Key word search: herbicide, aryloxyalkanoate dioxygenase, pyridyloxy auxin, 2, 4-D, bacillus thuringiensis, abiotic stress tolerance, insect resistance, phenoxy auxin, transgenic, transform, cassava vein mosaic virus promoter, fluroxypr, triclopyr.

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PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	28 MAR 2008
Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US06/42133	27 October 2006 (27.10.2006)	28 October 2005 (28.10.2005)

International Patent Classification (IPC) or both national classification and IPC

IPC(8): **A01H 5/00**(2006.01);**C12N 15/82**(2006.01);**C12N 5/04**(2006.01)
USPC: 800/295;800/300;800/301;800/302;800/278;800/289;435/468;435/419

Applicant

DOW AGROSCIENCES LLC

1. This opinion contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☒	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(*b*) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	01 December 2007 (01.12.2007)	Vinod Kumar Telephone No. (571) 272-4445

Form PCT/ISA/237 (cover sheet) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☒ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☒ on paper

☒ in electronic form

c. time of filing/furnishing

☒ contained in the international application as filed.

☒ filed together with the international application in electronic form.

☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:
   - ☐ paid additional fees
   - ☐ paid additional fees under protest and, where applicable, the protest fee
   - ☐ paid additional fees under protest but the applicable protest fee was not paid
   - ☒ not paid additional fees
2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
   - ☐ complied with
   - ☒ not complied with for the following reasons:

   See the lack of unity section of the International Search Report(Form PCT/ISA/210)

4. Consequently, this opinion has been established in respect of the following parts of the international application:
   - ☐ all parts.
   - ☒ the parts relating to claims Nos. 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-94 (SEQ ID NOs: 1 and 2)

Form PCT/ISA/237 (Box No. IV) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO
Inventive step (IS)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO
Industrial applicability (IA)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO

2. Citations and explanations:

Please See Continuation Sheet

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 19-21, 30, 73, and 75 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims 19-21, 30, 73, and 75 not fully supported by the description. The application, as originally filed, did not describe genus of sequences that would hybridize to SEQ ID NO: 1. These claims encompass structures whose structures are not described in the specification, and thus one skilled in the art would not know their function. The stringent conditions described on page 22 of specification would encompass hybridization of unrelated nucleic acid sequences to instant SEQ ID NO: 1.

Claims 19-21, 30, 73, and 75 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because of the claims 19-21, 30, 73, and 75 are not fully supported by the description. The description does not disclose the claimed invention in a manner sufficiently clear and complete for the claimed invention to be carried out by a person skilled in the art because the stringent conditions described on page 22 of specification would encompass hybridization of unrelated nucleic acid sequences to instant SEQ ID NO: 1. One skilled in the art would not know how to use said unrelated sequences or plants having said unrelated sequences.

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Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

V.1. Reasoned Statements:
The opinion as to Novelty was positive (Yes)with respect to claims 1-4, 42-43, 60-64, 77, 79-81, 83-84
The opinion as to Novelty was negative (No) with respect to claims 15-21, 30-32, 73-76
The opinion as to Inventive Step was positive (Yes)with respect to claims 1-4, 60-64, 79-81, 83-84
The opinion as to Inventive Step was negative(NO) with respect to claims 15-21, 30-32, 42-43, 73-77
The opinion as to Industrial Applicability was positive (YES) with respect to claims 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, 83-84
The opinion as to Industrial Applicability was negative(NO) with respect to claims NONE

V. 2. Citations and Explanations:

Claims 1-4, 42-43, 60-64, 77, 79-81, and 83-84 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a transgenic plant cell plant or seed derived thereof, comprising a polynucleotide that encodes an AAD-12 protein of SEQ ID NO: 2, or a variant of SEQ ID NO: 2 having aryloxyalkanoate dioxygenase activity and exhibiting at least 95% sequence identity to SEQ ID NO: 2. The prior art does not teach or fairly suggest that said plant further comprises a gene for insect or stress resistance. The prior art does not teach or suggest an isolated polynucleotide that encodes a protein that enzymatically degrades a phenoxy auxin or pyridyloxy auxin herbicide, and wherein said polynucleotide is operably linked with a cassava vein mosaic virus promoter.

Claims 15-21, 30-32, 73-76 lack novelty under PCT Article 33(2) as being anticipated by Kaphammer et al. (US Patent No. 5,608,147, Issued March 4, 1997).

Kaphammer et al. teach a transgenic plant cell or transgenic plant comprising a polynucleotide encoding a polypeptide tfdA which confers resistance to phenoxy auxin herbicide, such as 2-4-D. The reference also teaches an expression cassette comprising said polynucleotide operably linked to a 35S promoter or a plant, and wherein said expression cassette is used in obtaining said transgenic plant. See in particular, abstract; figures 1-7; columns 2-22.

The property of conferring resistance to a pyridyloxyl auxin herbicide would be inherent to the polynucleotide disclosed in the reference.

Kaphammer et al. anticipates the instantly claimed invention because the stringent conditions (see page 22 of specification) recited in claims 19-21, 30, 73, and 75 would allow hybridization of the polynucleotide taught by Kaphammer et al. The reference further teaches that said transgenic plant cell or plant would comprise more than one copy of the transgene (an additional herbicide resistant gene).

Thus Kaphammer et al. anticipated the claimed invention.

Claim 42 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Perlak et al. (PNAS, 88:3324-3328, 1991).

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach insect resistant gene.

Perlak et al. teach an insect resistant gene isolated from *Bacillus thuringiensis* and its expression in a transgenic plant to produce an insect resistant transgenic plant. See page 3324, abstract; page 3325, table 1; page 3327, figure 2, table 2.

It would have been obvious and within the scope of an ordinary skill in the art to introduce an insect resistant gene of Perlak et

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Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

al. in Kaphammer et al. transgenic plants using any method of plant transformation including the one taught by Kaphammer et al. or Perlak. One of ordinary skill in the art would have been motivated to do so for the purposes of obtaining transgenic plants that are resistant to insects and herbicides.

Claim 43 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Kasuga et al. (Nature Biotechnology, 17:287-291, 1999).

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach a stress tolerance gene.

Kasuga et al. teach DREB1A gene overexpressed in a transgenic plant to produce drought, salt and freezing tolerant transgenic plant. See in particular, abstract; page 288, figures 1 and 2; page 289, figures 3-5.

It would have been obvious and within the scope of an ordinary skill in the art to introduce a stress tolerant gene of Kasuga et al. in Kaphammer et al. transgenic plants using any method of plant transformation including the one taught by Kaphammer et al. or Perlak. One of ordinary skill would have been motivated to do so for the purposes of obtaining transgenic plants that are resistant herbicides and exhibit tolerance to abiotic stresses.

Claim 77 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Verdaguer et al. (Plant Molecular Biology, 31:1129-1139, 1996)

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach a cassava vein mosaic virus promoter.

Verdaguer et al. teach a cassava vein mosaic virus promoter and its use in high level expression of a coding sequence of interest in a transgenic plant. See in particular, page 1129, abstract; pages 1130-1132, materials and methods.

It would have been obvious and within the scope of an ordinary skill in the art to replace the promoter used in expressing Kaphammer et al. polynucleotide in the transgenic plant with Verdaguer et al. cassava vein mosaic virus promoter. One of ordinary skill would have been motivated to do so for the purposes of obtaining transgenic plants expressing high levels of the transgenic protein, and thereby impart higher resistance to herbicides in said transgenic plants with reasonable expectation of success.

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(54) Title: NOVEL HERBICIDE RESISTANCE GENES

(57) Abstract: The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide tolerant crop trait and selectable marker opportunities.

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NOVEL HERBICIDE RESISTANCE GENES

Background of the Invention

[0001] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0002] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant soybeans, for example, can be sprayed to control weeds without significantly damaging the soybean plants.

[0003] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. Alfalfa was the first perennial GTC introduced, furthering the opportunity for repeated use of glyphosate on the same crop and fields repeatedly over a period of years. More GTCs (*e.g.,* wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (*e.g.*, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; *see* "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0004] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection

pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

[0005] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Powles and Preston, 2006, Ng *et al.*, 2003; Simarmata *et al.*, 2003; Lorraine-Colwill *et al.*, 2003; Sfiligoj, 2004; Miller *et al.*, 2003; Heap, 2005; Murphy *et al.*, 2002; Martin *et al.*, 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past five years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Sorghum halepense, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis, Plantago lanceolata, Amaranthus palmerii, and Amaranthus rudis.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0006] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting

no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2005).

[0007] 2,4-D is in the phenoxy acid class of herbicides, as is MCPA. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known. Triclopyr and fluroxypyr are pyridyloxyacetic acid herbicides whose mode of action is as a synthetic auxin, also.

[0008] These herbicides have different levels of selectivity on certain plants (*e.g.,* dicots are more sensitive than grasses). Differential metabolism by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

[0009] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D currently formulated has a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0010] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0011] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many

homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0012] One unique example with low homology to *tfdA* (31% amino acid identity) is *sdpA* from *Delftia acidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002, Westendorf *et al., 2003*). This enzyme has been shown to catalyze the first step in (S)-dichlorprop (and other (S)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Transformation of this gene into plants, has not heretofore been reported.

[0013] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0014] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxyacetate auxins (such as 2,4-D and dichlorprop), pyridyloxyacetate auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. A multifunctional enzyme for the degradation of herbicides covering multiple modes of action has recently been described (PCT US/2005/014737; filed May 2, 2005). Another unique multifunctional enzyme and potential uses are described hereafter.

Brief Summary of the Invention

[0015] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of

the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, ALS- (imidazolinone, sulfonylurea), aryloxyalkanoate-, HPPD-, PPO-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0016] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, MCPA, triclopyr, fluroxypyr, and one or more commercially available herbicides (*e.g.*, glyphosate, glufosinate, paraquat, ALS-inhibitors (e.g., sulfonylureas, imidazolinones, triazolopyrimidine sulfonanilides, et al), HPPD inhibitors (e.g, mesotrione, isoxaflutole, *et al.*), dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0017] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide-tolerant crop (HTC) trait and selectable marker opportunities. Plants of the subject invention can be resistant throughout their entire life cycle.

[0018] There was no prior motivation to produce plants comprising an *AAD-12* gene (preferably an *AAD-12* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-12 enzyme to render the plants resistant a phenoxyacetic acid herbicide (such as 2,4-D) and/or one or more pyridyloxyacetates herbicides such as triclopyr and fluroxypyr. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0019] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxyacetate auxin and/or pyridyloxyacetates auxin herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid and other aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more pyridyloxyacetate or phenoxyacetate auxin herbicides to plants comprising an *AAD-12* gene. The subject invention also provides methods of using an *AAD-12* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-12*, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0020] **Figure 1** illustrates the general chemical reaction that is catalyzed by AAD-12 enzymes of the subject invention.

[0021] **Figure 2** is an amino acid sequence alignment of an exemplified AAD-12 protein, TfdA, AAD-2, AAD-1, and TauD.

[0022] **Figure 3** illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop.

Brief Description of the Sequences

[0023] **SEQ ID NO:1** is the nucleotide sequence of *AAD-12* from *Delftia acidovorans*.

[0024] **SEQ ID NO:2** is the translated protein sequence encoded by SEQ ID NO:1.

[0025] **SEQ ID NO:3** is the plant optimized nucleotide sequence of *AAD-12 (v1)*.

[0026] **SEQ ID NO:4** is the translated protein sequence encoded by SEQ ID NO:3.

[0027] **SEQ ID NO:5** is the E.coli optimized nucleotide sequence of *AAD-12 (v2)*.

[0028] **SEQ ID NO:6** is the sequence of the M13 forward primer.

[0029] **SEQ ID NO:7** is the sequence of the M13 reverse primer.

[0030] **SEQ ID NO:8** is the sequence of the forward *AAD-12 (v1)* PTU primer.

[0031] **SEQ ID NO:9** is the sequence of the reverse *AAD-12 (v1)* PTU primer.

[0032] **SEQ ID NO:10** is the sequence of the forward *AAD-12 (v1)* coding PCR primer.

[0033] **SEQ ID NO:11** is the sequence of the reverse *AAD-12 (v1)* coding PCR primer.

[0034] **SEQ ID NO:12** shows the sequence of the "sdpacodF" *AAD-12 (v1)* primer.

[0035] **SEQ ID NO:13** shows the sequence of the "sdpacodR" *AAD-12 (v1)* primer.

[0036] **SEQ ID NO:14** shows the sequence of the "Nco1 of Brady" primer.

[0037] **SEQ ID NO:15** shows the sequence of the "Sac1 of Brady" primer.

Detailed Description of the Invention

[0038] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of the subject herbicide tolerance trait for 2,4-D is its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the *AAD-12* gene is that unlike all *tfdA* homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (*e.g.*, triclopyr, fluroxypyr) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). *See* **Table 1**. A general illustration of the chemical reactions catalyzed by the subject AAD-12 enzyme is shown in **Figure 1**. (Addition of O_2 is stereospecific; breakdown of intermediate to phenol and glyoxylate is spontaneous.) It should be understood that the chemical structures in Figure 1 illustrate the molecular backbones and that various R groups and the like (such as those

shown in Table 1) are included but are not necessarily specifically illustrated in Figure 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the *AAD-12* gene in plants affords protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled. The subject invention can also be used to protect from drift or other off-site synthetic auxin herbicide injury for the full breadth of commercially available phenoxy auxins. **Table 1** defines commercially available pyridyloxy and phenoxy auxins and provides relevant chemical structures.

Table 1. Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

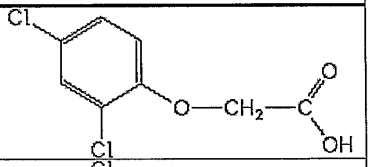
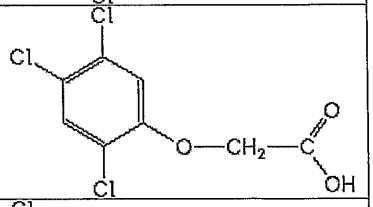
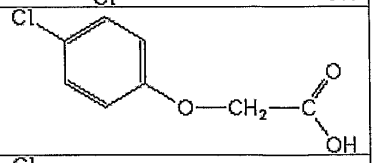
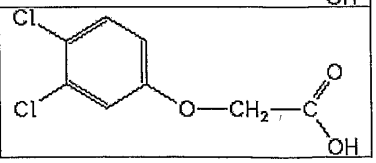
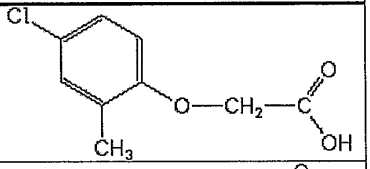
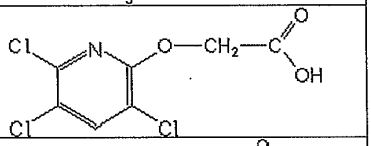
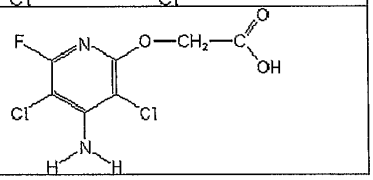
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	

Table 1. Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
MCPA	94-74-6	25 – 4000	125 - 1550	
Triclopyr	55335-06-3	50 – 2000	70 – 840	
Fluroxypyr	69377-81-7	25 – 2000	35 – 560	

[0039] A single gene (*AAD-12*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-12* can provide protection *in planta* to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing *AAD-12* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

[0040] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-12* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (*e.g.*, 2,4-D) and pyridyloxyacetates auxin herbicides (*e.g.*, triclopyr)—selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0041] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/*AAD-12* stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0042] Plantlets are typically resistant throughout the entire growing cycle. Transformed plants will typically be resistant to new herbicide application at any time the gene is expressed. Tolerance is shown herein to 2,4-D across the life cycle using the constitutive promoters tested thus far (primarily CsVMV and AtUbi 10). One would typically expect this, but it is an improvement upon other non-metabolic activities where tolerance can be significantly impacted by the reduced expression of a site of action mechanism of resistance,

for example. One example is Roundup Ready cotton, where the plants were tolerant if sprayed early, but if sprayed too late the glyphosate concentrated in the meristems (because it is not metabolized and is translocated); viral promoters Monsanto used are not well expressed in the flowers. The subject invention provides an improvement in these regards.

[0043] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants, surfactants, drift retardants, or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0044] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS,* glyphosate oxidoreductase (*GOX*), *GAT*), glufosinate resistance (*e.g.*, *Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g., PPO-1*), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), dicamba-degrading enzymes (*see, e.g.,* US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes. *In vivo* modified EPSPS can be used in some preferred embodiments, as well as Class I, Class II, and Class III glyphosate resistance genes.

[0045] Regarding additional herbicides, some additional preferred ALS inhibitors include but are not limited to the sulfonylureas (such as chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron), imidazoloninones (such as imazamox, imazethapyr, imazaquin), triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam),

pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include but are not limited to mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include but are not limited to flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0046] Additionally, *AAD-12* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0047] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-1 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

[0048] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0049] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and

pyridyloxyacetate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic acids, and pyridyloxyacetic acid herbicides by a recombinantly expressed AAD-12 enzyme.

[0050] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP *in vitro*. An additional advantage that *AAD-12* transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0051] The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an *AAD-12* gene.

[0052] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0053] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0054] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-

ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0055] The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, *sdpA*, from *Delftia acidivorans* (Westendorf *et al.*, 2002, 2003) with low homology to *tfdA* (31% amino acid identity). This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. *SdpA* has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0056] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

[0057] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and/or pyridyloxy auxin herbicides.

[0058] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. *AAD-12* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits [*e.g.*, glyphosate resistance, glufosinate resistance, ALS-inhibitor (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide) resistance, bromoxynil resistance, HPPD-

inhibitor resistance, PPO-inhibitor resistance, *et al.*], and insect resistance traits (*Cry1F, Cry1Ab, Cry 34/45*, other Bt. Proteins, or insecticidal proteins of a non-*Bacillis* origin, *et al.*) for example. Additionally, *AAD-12* can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0059] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-12*-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention.

[0060] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[0061] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

[0062] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[0063] The transgenic expression of the subject *AAD-12* genes is exemplified in, for example, *Arabidopsis*, tobacco, soybean, cotton, rice, corn and canola. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as pasture grasses or turf grass) and dicot

crops like alfalfa, clover, tree species, *et al.* Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

[0064] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

[0065] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

[0066] Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention

are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[0067] One cannot easily discuss the term "resistance" and not use the verb "tolerate" or the adjective "tolerant." The industry has spent innumerable hours debating Herbicide Tolerant Crops (HTC) versus Herbicide Resistant Crops (HRC). HTC is a preferred term in the industry. However, the official Weed Science Society of America definition of resistance is "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." As used herein unless otherwise indicated, herbicide "resistance" is heritable and allows a plant to grow and reproduce in the presence of a typical herbicidally effective treatment by a herbicide for a given plant, as suggested by the current edition of *The Herbicide Handbook* as of the filing of the subject disclosure. As is recognized by those skilled in the art, a plant may still be considered "resistant" even though some degree of plant injury from herbicidal exposure is apparent. As used herein, the term "tolerance" is broader than the term "resistance," and includes "resistance" as defined herein, as well an improved capacity of a particular plant to withstand the various degrees of herbicidally induced injury that typically result in wild-type plants of the same genotype at the same herbicidal dose.

[0068] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

[0069] The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[0070] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[0071] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria.

[0072] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutant strains can also be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[0073] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[0074] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook *et al.*, 1989, which is incorporated herein by reference.

[0075] Polynucleotides and probes. The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[0076] The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-12* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage. In addition, desired levels and times of expression can also depend on the type of plant and the level of tolerance desired. Some preferred embodiments use strong constitutive promoters combined with transcription enhancers and the like to increase expression levels and to enhance tolerance to desired levels. Some such applications are discussed in more detail below, before the Examples section.

[0077] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or

"coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[0078] In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

[0079] Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

[0080] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

[0081] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[0082] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[0083] Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

[0084] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

Tm = 81.5° C + 16.6 Log[Na+] + 0.41(%G+C) - 0.61(%formamide) - 600/length of duplex in base pairs.

[0085] Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

[0086] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

Tm (°C) = 2(number T/A base pairs) + 4(number G/C base pairs)

(Suggs *et al.*, 1981).

[0087] Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

[0088] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low:	1 or 2x SSPE, room temperature
Low:	1 or 2x SSPE, 42° C
Moderate:	0.2x or 1x SSPE, 65° C
High:	0.1x SSPE, 65° C.

[0089] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[0090] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.*, 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[0091] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the

scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[0092] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein.

[0093] The top two results of BLAST searches with the native aad-12 nucleotide sequence show a reasonable level of homology (about 85%) over 120 basepairs of sequence. Hybridization under certain conditions could be expected to include these two sequences. *See* GENBANK Acc. Nos. DQ406818.1 (89329742; *Rhodoferax*) and AJ6288601.1 (44903451; *Sphingomonas*). *Rhodoferax* is very similar to *Delftia* but *Sphingomonas* is an entirely different Class phylogenetically.

[0094] The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[0095] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused

fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[0096] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[0097] Specific changes to the "active site" of the enzyme can be made to affect the inherent functionallity with respect to activity or stereospecificity (see alignment **Figure 2**). Muller *et. al.* (2006). The known tauD crystal structure was used as a model dioxygenase to determine active site residues while bound to its inherent substrate taurine. Elkins *et al.* (2002) "X-ray crystal structure of *Escerichia coli* taurine/alpha-ketoglutarate dioxygenase complexed to ferrous iron and substrates," *Biochemistry* 41(16):5185-5192. Regarding sequence optimization and designability of enzyme active sites, *see* Chakrabarti *et al.*, PNAS, (Aug. 23, 2005), 102(34):12035-12040.

[0098] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190,

191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, or 293 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[0099] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[00100] It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.*, Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term "protein" can include functionally active truncations.

[00101] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically

encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[00102] Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[00103] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.*, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[00104] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby

an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 2** provides a listing of examples of amino acids belonging to each class.

Table 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[00105] In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

[00106] As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

[00107] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

[00108] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or

monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

[00109] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf, legume forages (e.g., alafalfa and clover), pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[00110] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[00111] Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[00112] Although plants can be preferred, the subject invention also includes production of highly active recombinant AAD-12 in a *Pseudomonas fluorescens* (Pf) host strain, for example. The subject invention includes preferred growth temperatures for maintaining soluble active AAD-12 in this host; a fermentation condition where AAD-12 is produced as more than 40% total cell protein, or at least 10 g/L; a purification process results high recovery of active recombinant AAD-12 from a Pf host; a purification scheme which yields at least 10 g active AAD-12 per kg of cells; a purification scheme which can yield 20 g active

AAD-12 per kg of cells; a formulation process that can store and restore AAD-12 activity in solution; and a lyophilization process that can retain AAD-12 activity for long-term storage and shelf life.

[00113] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[00114] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[00115] Vectors comprising an *AAD-12* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc.* Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli.* The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

[00116] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle

bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria.* The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria.* They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[00117] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00118] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[00119] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate (*e.g., PAT/bar*), glyphosate (*EPSPS*), ALS-inhibitors (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, *et al.*), bromoxynil, HPPD-inhibitor resistance, PPO-inhibitors, ACC-ase inhibitors , and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00120] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. *See* U.S. Patent No. 6,809,232. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to

Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00121] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[00122] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00123] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation

thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

[00124] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00125] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G41;, hygromycin resistance; methotrexate resistance, as well as those genes which encode for resistance or tolerance to glyphosate; phosphinothricin (bialaphos or glufosinate); ALS-inhibiting herbicides (imidazolinones, sulfonylureas and triazolopyrimidine herbicides), ACC-ase inhibitors (e.g., ayryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

[00126] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-

glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00127] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00128] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such

include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00129] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00130] Means of further increasing tolerance or resistance levels. It is shown herein that plants of the subject invention can be imparted with novel herbicide resistance traits without observable adverse effects on phenotype including yield. Such plants are within the scope of the subject invention. Plants exemplified and suggested herein can withstand 2X, 3X, 4X, and 5X typical application levels, for example, of at least one subject herbicide. Improvements in these tolerance levels are within the scope of this invention. For example, various techniques are know in the art, and can forseeably be optimized and further developed, for increasing expression of a given gene.

[00131] One such method includes increasing the copy number of the subject AAD-12 genes (in expression cassettes and the like). Transformation events can also be selected for those having multiple copies of the genes.

[00132] Strong promoters and enhancers can be used to "supercharge" expression. Examples of such promoters include the preferred 35T promoter which uses 35S enhancers. 35S, maize ubiquitin, Arabidopsis ubiquitin, A.t. actin, and CSMV promoters are included for such uses. Other strong viral promoters are also preferred. Enhancers include 4 OCS and the 35S double enhancer. Matrix attachment regions (MARs) can also be used to increase transformation efficiencies and transgene expression, for example.

[00133] Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

[00134] Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See *e.g.* U.S. Patent No. 7,058,515; Larson et al., *Protein Sci.* 2002 11: 2804-2813, "Thoroughly sampling sequence space: Large-scale protein design of structural ensembles."; Crameri *et al.*, *Nature Biotechnology* 15, 436 - 438 (1997), "Molecular evolution of an arsenate detoxification pathway by DNA shuffling."; Stemmer, W.P.C.1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc. Natl. Acad. Sci. USA 91: 10747–10751; Stemmer, W.P.C.1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature 370: 389–391; Stemmer, W.P.C.1995. Searching sequence space. Bio/Technology 13: 549–553; Crameri, A., Cwirla, S. and Stemmer, W.P.C.1996. Construction and evolution of antibody-phage libraries by DNA shuffling. Nature Medicine 2: 100–103; and Crameri, A., Whitehorn, E.A., Tate, E. and Stemmer, W.P.C. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnology 14: 315–319.

[00135] The activity of recombinant polynucleotides inserted into plant cells can be dependent upon the influence of endogenous plant DNA adjacent the insert. Thus, another option is taking advantage of events that are known to be excellent locations in a plant genome for insertions. *See e.g.* WO 2005/103266 A1, relating to cry1F and cry1Ac cotton events; the subject AAD-12 gene can be substituted in those genomic loci in place of the cry1F and/or cry1Ac inserts. Thus, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (USPA 20030232410), relating to the use of zinc fingers for targeted recombination. The use of recombinases (cre-lox and flp-frt for example) is also known in the art.

[00136] AAD-12 detoxification is believed to occur in the cytoplasm. Thus, means for further stabilizing this protein and mRNAs (including blocking mRNA degradation) are included in aspects of the subject invention, and art-known techniques can be applied accordingly. The subject proteins can be designed to resist degradation by proteases and the like (protease cleavage sites can be effectively removed by re-engineering the amino acid sequence of the protein). Such embodiments include the use of 5' and 3' stem loop structures like UTRs

from osmotin, and per5 (AU-rich untranslated 5' sequences). 5' caps like 7- methyl or 2'-O-methyl groups, e.g., 7-methylguanylic acid residue, can also be used. *See, e.g.,*: Proc. Natl. Acad. Sci. USA Vol. 74, No. 7, pp. 2734-2738 (July 1977) *Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis.* Protein complexes or ligand blocking groups can also be used.

[00137] Computational design of 5' or 3' UTR most suitable for AAD-12 (synthetic hairpins) can also be conducted within the scope of the subject invention. Computer modeling in general, as well as gene shuffling and directed evolution, are discussed elsewhere herein. More specifically regarding computer modeling and UTRs, computer modeling techniques for use in predicting/evaluating 5' and 3' UTR derivatives of the present invention include, but are not limited to: MFold version 3.1 available from Genetics Corporation Group, Madison, WI (see Zucker et al., Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In *RNA Biochemistry and Biotechnology*, 11-43, J. Barciszewski & B.F.C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, NL, (1999); Zucker *et al.*, *Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure. J. Mol. Biol.* 288, 911-940 (1999); Zucker *et al.*, RNA Secondary Structure Prediction.In *Current Protocols in Nucleic Acid Chemistry* S. Beaucage, D.E. Bergstrom, G.D. Glick, and R.A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, (2000)), COVE (RNA structure analysis using covariance models (stochastic context free grammar methods)) v.2.4.2 (Eddy & Durbin, Nucl. Acids Res. 1994, 22: 2079-2088) which is freely distributed as source code and which can be downloaded by accessing the website genetics.wustl.edu/eddy/software/, and FOLDALIGN, also freely distributed and available for downloading at the website bioinf.au.dk. FOLDALIGN/ (see *Finding the most significant common sequence and structure motifs in a set of RNA sequences.* J. Gorodkin, L. J. Heyer and G. D. Stormo. Nucleic Acids Research, Vol. 25, no. 18 pp 3724-3732, 1997; *Finding Common Sequence and Structure Motifs in a set of RNA Sequences.*. J. Gorodkin, L. J. Heyer, and G. D. Stormo. ISMB 5;120-123, 1997).

[00138] Embodiments of the subject invention can be used in conjunction with naturally evolved or chemically induced mutants (mutants can be selected by screening techniques, then transformed with AAD-12 and possibly other genes). Plants of the subject invention can be combined with ALS resistance and/or evolved glyphosate resistance. Aminopyralid resistance, for example, can also be combined or "stacked" with an AAD-12 gene.

[00139] Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve desired traits.

[00140] Further improvements also include use with appropriate safeners to further protect plants and/or to add cross resistance to more herbicides. (Safeners typically act to increase plants immune system by activating/expressing cP450. Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism, without compromising weed control efficacy.)

[00141] Herbicide safeners include benoxacor, cloquintocet, cyometrinil, dichlormid, dicyclonon, dietholate, fenchlorazole, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen, mefenpyr, mephenate, naphthalic anhydride, and oxabetrinil. Plant activators (a new class of compounds that protect plants by activating their defense mechanisms) can also be used in embodiments of the subject invention. These include acibenzolar and probenazole.

[00142] Commercialized safeners can be used for the protection of large-seeded grass crops, such as corn, grain sorghum, and wet-sown rice, against preplant-incorporated or preemergence-applied herbicides of the thiocarbamate and chloroacetanilide families. Safeners also have been developed to protect winter cereal crops such as wheat against postemergence applications of aryloxyphenoxypropionate and sulfonylurea herbicides. The use of safeners for the protection of corn and rice against sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides is also well-established. A safener-induced enhancement of herbicide detoxification in safened plants is widely accepted as the major mechanism involved in safener action. Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione *S*-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. Hatzios KK, Burgos N (2004) "Metabolism-based herbicide resistance: regulation by safeners," Weed Science: Vol. 52, No. 3 pp. 454–467.

[00143] Use of a cytochrome p450 monooxygenase gene stacked with AAD-12 is one preferred embodiment. There are P450s involved in herbicide metabolism; cP450 can be of mammalian or plant origin, for example. In higher plants, cytochrome P450 monooxygenase (P450) is known to conduct secondary metabolism. It also plays an important role in the oxidative metabolism of xenobiotics in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase). Resistance to some herbicides has been reported as a result of the metabolism by P450 as well as glutathione S-transferase. A number of microsomal P450

species involved in xenobiotic metabolism in mammals have been characterized by molecular cloning. Some of them were reported to metabolize several herbicides efficiently. Thus, transgenic plants with plant or mammalian P450 can show resistance to several herbicides.

[00144] One preferred embodiment of the foregoing is the use cP450 for resistance to acetochlor (acetochlor-based products include Surpass®, Keystone®, Keystone LA, FulTime® and TopNotch® herbicides) and/or trifluralin (such as Treflan®). Such resistance in soybeans and/or corn is included in some preferred embodiments. For additional guidance regarding such embodiments, *see e.g.* Inui *et al.*, "A selectable marker using cytochrome P450 monooxygenases for Arabidopsis transformation," *Plant Biotechnology* 22, 281–286 (2005) (relating to a selection system for transformation of Arabidopsis thaliana via Agrobacterium tumefaciens that uses human cytochrome P450 monooxygenases that metabolize herbicides; herbicide tolerant seedlings were transformed and selected with the herbicides acetochlor, amiprophos-methyl, chlorpropham, chlorsulfuron, norflurazon, and pendimethalin); Siminszky et al., "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides," *PNAS* Vol. 96, Issue 4, 1750-1755, February 16, 1999; Sheldon et al, *Weed Science*: Vol. 48, No. 3, pp. 291–295, "A cytochrome P450 monooxygenase cDNA (CYP71A10) confers resistance to linuron in transgenic Nicotiana tabacum"; and "Phytoremediation of the herbicides atrazine and metolachlor by transgenic rice plants expressing human CYP1A1, CYP2B6, and CYP2C19," *J Agric Food Chem.* 2006 Apr 19;54(8):2985-91 (relating to testing a human cytochrome p450 monooxygenase in rice where the rice plants reportedly showed high tolerance to chloroacetomides (acetochlor, alachlor, metoachlor, pretilachlor, and thenylchlor), oxyacetamides (mefenacet), pyridazinones (norflurazon), 2,6-dinitroanalines (trifluralin and pendimethalin), phosphamidates (amiprofos-methyl, thiocarbamates (pyributicarb), and ureas (chlortoluron)).

[00145] There is also the possibility of altering or using different 2,4-D chemistries to make the subject AAD-12 genes more efficient. Such possible changes include creating better substrates and better leaving groups (higher electronegativity).

[00146] Auxin transport inhibitors (e.g. diflufenzopyr) can also be used to increase herbicide activity with 2,4-D.

[00147] Unless specifically indicated or implied, the terms "a", "an", and "the" signify "at least one" as used herein.

[00148] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00149] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Method for Identifying Genes That Impart Resistance to 2,4-D *In Planta*

[00150] As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (*i.e.*, α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with ≤ 50% homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to *tfdA* from *Ralstonia eutropha)* can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00151] A single gene (*sdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 31% amino acid identity to *tfdA*. Percent identity was determined by first translating both the *sdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

Example 2 – Optimization of Sequence for Expression in Plants and Bacteria

[00152] 2.1 – Background.

[00153] To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[00154] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00155] **Table 3** illustrates how high the G+C content is in maize. For the data in **Table 3**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

Table 3: Compilation of G + C contents of protein coding regions of maize genes

Protein Class[a]	Range % G + C	Mean % G + C[b]
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)[c]

[a] Number of genes in class given in parentheses.
[b]Standard deviations given in parentheses.
[c] Combined groups mean ignored in mean calculation

[00156] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[00157] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 4**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new

sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, *etc.* with the next preferred codon of choice.

Table 4. Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

[00158] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and

about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00159] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

[00160] 2.2 – *AAD-12* Plant rebuild analysis.

[00161] Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native *AAD-12* coding region (SEQ ID NO:1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 (*AAD-12*) is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence *AAD-12 (v1)* (SEQ ID NO:3) was developed that encodes a protein (SEQ ID NO:4) which is the same as the native SEQ ID NO:2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO:4). The additional alanine codon (GCT; underlined in SEQ ID NO:3) encodes part of an *NcoI* restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical. **Table 5** shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences

(Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

Table 5. Codon composition comparisons of coding regions of Native *AAD-12*, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #	Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #
ALA (A)	GCA	1	10	11	LEU (L)	CTA	0	0	0
	GCC	35	16	15		CTC	1	8	8
	GCG	7	0	0		CTG	23	0	0
	GCT	0	18	17		CTT	0	8	8
ARG (R)	AGA	0	4	5		TTA	0	0	0
	AGG	0	4	6		TTG	0	8	8
	CGA	0	0	0	LYS (K)	AAA	1	1	2
	CGC	15	6	4		AAG	5	5	4
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	4	3	PHE (F)	TTC	7	5	5
ASN (N)	AAC	3	2	2		TTT	1	3	3
	AAT	1	2	2	PRO (P)	CCA	0	5	6
ASP (D)	GAC	15	9	9		CCC	9	4	4
	GAT	2	8	8		CCG	5	0	0
CYS (C)	TGC	3	2	2		CCT	0	5	5
	TGT	0	1	1	SER (S)	AGC	5	4	3
END	TAA	1	0	1		AGT	0	0	0
	TAG	0	0			TCA	0	3	3
	TGA	0	1			TCC	2	3	3
GLN (Q)	CAA	1	8	7		TCG	6	0	0
	CAG	13	6	7		TCT	0	3	3
GLU (E)	GAA	3	4	4	THR (T)	ACA	1	4	5
	GAG	8	7	7		ACC	11	7	7
GLY (G)	GGA	0	8	7		ACG	5	0	0
	GGC	24	7	7		ACT	1	7	6
	GGG	1	3	4	TRP (W)	TGG	8	8	8
	GGT	0	7	7	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	9	9		TAT	1	2	2
	CAT	8	7	7	VAL (V)	GTA	0	0	0
ILE (I)	ATA	0	2	2		GTC	6	8	7
	ATC	10	4	5		GTG	18	8	9
	ATT	1	5	4		GTT	0	8	8
	Totals	163	164	163		Totals	130	130	130

[00162] It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

[00163] 2.3 Rebuild for *E. coli* Expression

[00164] Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in *Escherichia coli and Salmonella typhimurium cellular and molecular biology, vol. 2*, pp. 2047–2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umbarger, H. (eds.) American Society for Microbiology, Washington, DC). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for these *E. coli*-Class II gene coding regions. It is thought that a protein coding region having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO:4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (*i.e.* Shine Dalgarno sequences) The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO:5 and encodes the protein disclosed in SEQ ID NO:4.

[00165] The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. Table 6 presents the codon compositions of the native *AAD-12* coding region (Columns A and D), an *AAD-12* coding region optimized for expression in *E. coli* (v2; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).

Table 6. Codon composition comparisons of coding regions of Native *AAD-12*, *E. coli*-Optimized version (v2) and a Theoretical *E. coli* Class II-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor. Class II #	Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor, Class II #
ALA (A)	GCA	1	13	13	LEU (L)	CTA	0	0	0
	GCC	35	0	0		CTC	1	2	0
	GCG	7	18	17		CTG	23	20	24
	GCT	0	13	14		CTT	0	1	0
ARG (R)	AGA	0	0	0		TTA	0	1	0
	AGG	0	0	0		TTG	0	0	0
	CGA	0	0	0	LYS (K)	AAA	1	4	5
	CGC	15	6	6		AAG	5	2	1
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	12	12	PHE (F)	TTC	7	6	6
ASN (N)	AAC	3	4	4		TTT	1	2	2
	AAT	1	0	0	PRO (P)	CCA	0	3	2
ASP (D)	GAC	15	10	9		CCC	9	0	0
	GAT	2	7	8		CCG	5	11	12
CYS (C)	TGC	3	2	2		CCT	0	0	0
	TGT	0	1	1	SER (S)	AGC	5	4	4
END	TAA	1	1	1		AGT	0	0	0
	TAG	0	0	0		TCA	0	0	0
	TGA	0	0	0		TCC	2	5	4
GLN (Q)	CAA	1	3	3		TCG	6	0	0
	CAG	13	11	11		TCT	0	4	5
GLU (E)	GAA	3	8	8	THR (T)	ACA	1	0	0
	GAG	8	3	3		ACC	11	12	12
GLY (G)	GGA	0	0	0		ACG	5	0	0
	GGC	24	12	11		ACT	1	6	6
	GGG	1	0	0	TRP (W)	TGG	8	8	8
	GGT	0	13	14	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	11	11		TAT	1	2	2
	CAT	8	5	5	VAL (V)	GTA	0	6	6
ILE (I)	ATA	0	0	0		GTC	6	0	0
	ATC	10	7	7		GTG	18	8	7
	ATT	1	4	4		GTT	0	10	11
	Totals	163	164	164		Totals	130	130	130

[00166] It is clear from examination of Table 6 that the native and *E. coli*-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

[00167] 2.4 – Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate

synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO:20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00168] A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 7. Columns D and H of Table 7 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 7. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

Weighted % of C1 =1/(%C1 + %C2 + %C3 + *etc.*) x %C1 x 100

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 7 (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of Table 7. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table

for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

Table 7. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	**Codon**	**Weighted %**	**Soybean %**	**Amino Acid**	**Codon**	**Weighted %**	**Soybean %**
ALA (A)	GCA	33.1	30.3	**LEU (L)**	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	**LYS (K)**	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	**6.0**	**MET (M)**	ATG	100.0	100
	CGT	16.9	14.5	**PHE (F)**	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	**PRO (P)**	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	**SER (S)**	AGC	16.0	15.1
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	**6.1**
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	**THR (T)**	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	**8.3**
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	**TRP (W)**	TGG	100.0	100
	GGT	30.4	30.4	**TYR (Y)**	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	**VAL (V)**	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

[00169] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 from PCT/US2005/014737 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00170] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is disclosed as bases 1-1575 of SEQ ID NO:22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO:22 of PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston TX).

Example 3-Cloning of expression and transformation vectors

[00171] 3.1 Construction of E.coli, pET expression vector.

[00172] Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba 1, Xho 1) *AAD-12 (v2)* was cut out of the picoscript vector, and ligated into a pET280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

[00173] To differentiate between *AAD-12 (v2)* : pET280 and pCR2.1 : pET280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB-S/S, and grown at 37° C for 4 hours with agitation. Each culture was then spotted onto LB + Kanamycin 50 μg/ml plates, which were incubated at 37° C overnight. Colonies that grew on the LB-K were assumed to

have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before, and checked for correctness with digestion by XbaI / XhoI. The final expression construct was given the designation pDAB3222.

[00174] 3.2- Construction of *Pseudomonas* expression vector

[00175] The *AAD-12 (v2)* open reading frame was initially cloned into the modified pET expression vector (Novagen), "pET280 S/S", as an XbaI – XhoI fragment. The resulting plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The *AAD-12 (v2)* open reading frame from pDAB725 was transferred into the *Pseudomonas* expression vector, pMYC1803, as an XbaI – XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB324 *Pseudomonas* expression strains.

[00176] 3.3 – Completion of binary vectors.

[00177] The plant optimized gene *AAD-12 (v1)* was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:6) and M13 Reverse (SEQ ID NO:7) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-12 (v1)* DNA sequence. The *AAD-12 (v1)* gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-12 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB724, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-12 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium* (see section 7.2).

[00178] 3.4 - Cloning of additional transformation constructs.

[00179] All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular

cloning methods (Maniatis *et al.,* 1982). **Table 8** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

Table 8. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
724	-	A, Ct, S	AAD12 v1	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3274	-	A	AAD12 v1	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	-	-	Agro binary
3278	1580	T	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2			Spectinomycin	-	pat	AtUbi10	Agro binary
3285	-	A	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3759	-	A, Ca, S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi10	Agro binary
4101	1863	Cn, R	AAD12 v1	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers / Gun
4464	-	S	AAD12 v1	CsVMV	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4468	-	S	AAD12 v1	AtUbi10	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4472	-	S	AAD12 v1	AtUbi3	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4476	-	S	AAD12 v1	ZmUbi1	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary
4480	-	S	AAD12 v1	AtAct2	-	RB7 Mar v2	-	-	Spectinomycin	-	pat	CsVMV	Agro binary

*A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
Atubi3= *Arabidopsis thaliana* Ubiquitin 3 Promoter
AtAct2= *Arabidopsis thaliana* Actin 2 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

Example 4 - Recombinant AAD-12 (v2) Expression and Purification in *Pseudomonas fluorescens*

[00180] 4.1- *Pseudomonas fluorescens* fermentation

[00181] For shake flask experiment, 200 µl of *Pseudomonas fluorescens* strain MB324 glycerol stock carried *AAD-12* (v2) construct pDAB739 (sec 3.2) was used to inoculate 50 ml fresh LB media supplemented with 30 µg/ml tetracycline/HCl. The culture (in a 250 ml baffled Erlenmeyer flask) was incubated on a shaker (New Brunswick Scientific Model Innova 44) at 300 rpm and 30 °C for 16 hrs. 20 ml of seed culture was transferred into 1 L *Pseudomonas fluorescens* culture media (Yeast extract, 5 g/L; K_2HPO_4, 5 g/L; $(NH_4)_2PO_4$, 7.5 g/L; $(NH_4)_2SO_4$; $MgSO_4$-$7H_2O$, 1 g/L; KCl, 0.5 g/L; $CaCl_2$-$2H_2O$, 0.5 g/L; NaCitrate-$2H_2O$, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: $FeCl_3$-$6H_2O$, 5.4 g/L; $MnCl_2$-$4H_2O$, 1 g/L; $ZnSO_4$-$7H_2O$, 1.45 g/L; $CuSO_4$-$5H_2O$, 0.25 g/L; H_3BO_3, 0.1 g/L; $(NH_4)_6MO_7O_{24}$, 0.1 g/L; concentrated HCl, 13 ml/L) supplemented with 20 µg/ml tetracycline/HCl and 250 µl of Pluronic L61(anti-foam) in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 30 °C and 300 rpm for 24 hrs. Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was added to 1 mM final in the cultures and continued to incubate for approximately 48 hrs at 25 °C. Cells were harvested by centrifugation at 7 krpm at 4 °C for 15 min, and cell paste was stored at -80 °C or immediately processed for purification.

[00182] For tank experiments, 1 ml each of the glycerol stock was inoculated a 1 L baffled flask containing 200 ml of LB media supplemented with 30 µg/ml tetracycline/HCl at 300 rpm and 32 °C for 16-24 hrs. The combined culture from three flasks (600 ml) was then aseptically transferred to a 20 L fermentor (B. Braun Bioreactor Systems) containing 10 L of Dow proprietary defined medium (through Teknova, Hollister, CA) designed to support high cell density growth. Growth temperature was maintained at 32 °C and the pH was controlled at the desired set-point through the addition of aqueous ammonia. Dissolved oxygen was maintained at a positive level in the liquid culture by regulating the sparged air flow and the agitation rates. The fed-batch fermentation process was carried out for approximately 24 hrs till cell density reached 170-200 OD_{575}. IPTG was added to 1 mM to induce the recombinant protein expression and the temperature was reduced and maintained to 25 °C using circulation of cold-water supply. The induction phase of the fermentation was allowed to continue for another 24 hrs. Samples (30 ml) were collected for various analyses to determine cell density

and protein expression level at 6, 12, and 18 hrs post-induction time points. At the end of a fermentation run, cells were harvested by centrifugation at 10 krpm for 30 min. The cell pellets were frozen at -80 °C for further processing.

[00183] 4.2- Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

[00184] Approximately 100-200 g of frozen (or fresh) *Pseudomonas* cells were thawed and resuspended in 1-2 L of extraction buffer containing 20 mM Tris-HCl, pH 8.5, and 25 ml of Protease inhibitor cocktail (Sigma cat# P8465). The cells were disrupted using Microfluidizer (model M110L or 110Y) (Microfluidics, Newton, MA) on ice with one pass at 11,000-12,000 psi. The lysate was centrifuged at 24,000 rpm for 20 min. The supernatant was transferred and dialyzed against 10 volumes of 20 mM Tris-HCl, pH 8.5 overnight at 4 °C, or diafiltrated with this buffer and filtered through a 0.45 μm membrane before applying to the column separations. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4 °C. Prior to loading, a Q Sepharose Fast Flow column (Pharmacia XK 50/00, 500 ml bed size) was equilibrated with 20 mM Tris-HCl, pH 8.5 buffer. The sample was applied to the column at 15 ml/min and then washed with this buffer until the eluate OD_{280} returned to baseline. Proteins were eluted with 2 L of linear gradient from 0 to 0.3 M NaCl at a flow rate of 15 ml/min, while 45 ml fractions were collected. Fractions containing AAD-12 activity as determined by the colorimetric enzyme assay and also corresponding to the predicted molecular weight of AAD-12 protein (about 32 kDa band on SDS-PAGE) were pooled. Solid ammonium sulfate to final 0.5 M was added to the sample, and then applied to a Phenyl HP column (Pharmacia XK 50/20, 250 ml bed size) equilibrated in 0.5 M ammonium sulfate in 20 mM Tris-HCl, pH 8.0. This column was washed with the binding buffer at 10 ml/min until the OD_{280} of the eluate returned to baseline, proteins were eluted within 2 column volumes at 10 ml/min by a linear gradient from 0.5 M to 0 Ammonium sulfate in 20 mM Tris-HCl, pH 8.0, and 12.5 ml fractions were collected. The main peak fractions containing AAD-12 were pooled, and if necessary, concentrated using a MWCO 10 kDa cut-off membrane centrifugal filter device (Millipore). In some cases the sample was further applied to a Superdex 75 gel filtration column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure AAD-12 were pooled and stored at -80 °C. In most cases, AAD-12 protein purity is approaching or above 99% after sequential ion-exchange column and

hydrophobic interaction column two-step separation. A typical yield for purified AAD-12 is 12-18 mg/g of wet cells. Bulk protein sample was formulated in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM DTT, and 1% Trehalose by diafiltration, and lyophilized on the Virtis Freezemobile Model 25EL (Virtis, Cardiner, NY) for long-term storage.

[00185] Protein concentration was initially measured by Bradford assay using Bio-Rad Protein assay kit (cat# 500-0006) with bovine serum albumin as standard. When needed, more accurate protein concentration was determined by using total amino acid hydrolysis. The sample was analyzed in Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) with amino acid calibration standards (cat# PN5061-3330) purchased from Agilent.

[00186] AAD-12 activity was determined through out the processes to ensure no loss of the enzyme activity by each treatment and manipulation, as described in the Example 5 below. Protein purity was monitored by using SDS-PAGE and analytical size exclusion chromatography. Purified protein sample was further verified and confirmed by N-terminal amino acid sequencing, and shown consisting of expected AQTTLQITPT residues at its N-terminus. Short and long-term protein stability was tested by enzymatic activity and by native-PAGE and SDS-PAGE gel analysis under both non-reducing and reducing conditions. And it was noticed that AAD-12 is prone to oligomerization via disulfide bond formation, therefore typically 2 mM DTT was used for protein storage. Phosphate-buffer saline (PBS) and Tris-buffer saline (TBS) were tested for protein lyophilization, with and without the presence of 1% trehalose. Additionally, the endotoxin and DNA contaminant context from purified sample were measured respectively, and the integrity of the AAD-12 protein was also assessed by isoelectric focusing (IEF) analysis.

[00187] Ten milligrams of purified AAD-12 (v2) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 ml of complete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification, and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

Example 5 – *In vitro* assays of AAD-12 activity

[00188] 5.1 – Assay via colorimetric phenol detection.

[00189] Enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2 μM phenol.

[00190] Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 5 minutes of incubation at 25° C, the reaction was terminated by addition of 30 μl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH) and 0.2 % 4-aminoantipyrine. Then 10 μl 0.8 % potassium ferricyanide was added and after 5 or 10 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols.

[00191] 5.2 – Assay via detection of chloropyridinol

[00192] AAD-12 action on potential substrates such as the herbicide triclopyr containing a substituted pyridine (rather than benzene rings) will release a pyridinol on cleavage of the aryloxyalkanoate bond. Pyridinols were not detected using the aminoantipyrine/ferricyanide phenol detection described in the preceding section. However, it was found that product chloropyridinols absorb strongly in the near UV with λ_{max} of 325 nm at pH 7 (extinction coefficient ~8,400 $M^{-1}.cm^{-1}$). This was used to create a continuous microplate-based spectrophotometric assay. Assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate,

the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero and the increase in absorbance followed for 10 minutes at 325 nm in a microplate reader. The first 2 minutes of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 µl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9 µM chloropyridinol.

[00193] 5.3 – Colorimetric assay using 2-(2-chloro,4-nitrophenoxy)propionate

[00194] A convenient assay of AAD-12 was devised using 2-(2-chloro,4-nitrophenoxy)propionate (CNPP) as substrate. Cleavage of CNPP by AAD-12 releases 2-chloro,4-nitrophenol. This phenol has a bright yellow absorbance at 410 nm at pH 7 enabling the reaction to be followed continuously or by endpoint analysis. The presence of AAD-12 activity can be monitored visually without the need for addition of further reagents. Microplate-based spectrophotometric assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 µM NH_4FeSO_4, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate amount of CNPP (added from a 10 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of CNPP, enzyme, or α-ketoglutarate at time zero and the increase in absorbance followed for 10 min at 410 nm in a microplate reader. The first 2 min of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 µl final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1 µM 2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be $K_m = 31 \pm 5.5$ µM and $k_{cat} = 16.2 \pm 0.79$ min^{-1}.

Example 6 – *In vitro* activity of AAD-12 on various substrates

[00195] 6.1 – AAD-12 (v2) activity on (R,S)- dichlorprop, (R)-dichlorprop, (S)-dichlorprop and 2,4-D

[00196] Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4 µg purified AAD-12 (v2). (R,S)-dichlorprop (R,S-DP) was tested at 1 mM and (R)-dichlorprop, (S)-dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in **Figure 3**, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4 µg AAD-12 (v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of α-

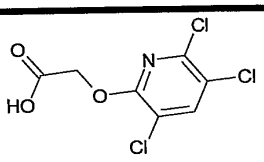
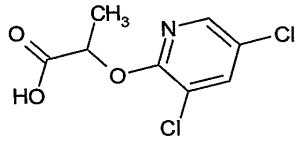
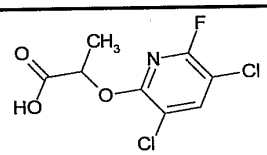
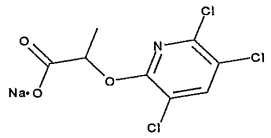
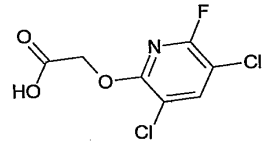
ketoglutarate. After 5 minutes, the reaction was quenched, and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

[00197] AAD-12 (v2) has excellent activity on (R,S)-dichlorprop and (S)-dichlorprop and has minimal activity on (R)-dichlorprop. This indicates that AAD-12 (v2) has a clear (S)-enantiomeric preference. The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)-dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively.

[00198] 6.2 – AAD-12 (v2) activity on pyridyloxyalkanoates

[00199] Using the pyridinol assay described in Example 5.2, five pyridyloxyalkanoates were assayed at 1 mM in a reaction mix containing 6.8 µg purified AAD-12 (v2). The rates of each reaction were monitored and are presented in Table 9. All five pyridyloxyalkanoates were cleaved to release pyridinols by AAD-12 (v2). The rates for the oxypropionate substrates 116844 and 91767 were somewhat faster than those for the corresponding acetates (triclopyr and 93833 respectively) indicating a preference of AAD-12 (v2) for oxypropionate over oxyacetate side chains. These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.

Table 9. Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8µg AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of α-ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without α-ketoglutarate was subtracted from the rates with substrate.

STRUCTURE	ID	Rate (mAU/min)	Rate relative to triclopyr
	triclopyr	97	1
	66357	225	2.3
	91767	190	0.8
	116844	257	1.4
	93833	118	0.5

[00200] 6.3 – Kinetic constants of AAD-12 (v2) for 2,4-D, (R,S)-DCP and triclopyr

[00201] The K_m and k_{cat} values of purified AAD-12 (v2) for the herbicides 2,4-D, (R,S)-dichlorprop and triclopyr were determined using the appropriate assay method. Substrate inhibition occurred at high concentrations (>1 mM) of 2,4-D and (R,S)-DCP so concentrations below this were used to fit the data to the Michaelis-Menten equation using

Grafit 4.0 (Erithacus Software, UK). No substrate inhibition was noted for triclopyr up to 2 mM. The kinetic constants are summarized in Table 10. From these data, the rate of AAD-12 (v2) cleavage of triclopyr is ~5% that of 2,4-D, under maximal velocity conditions.

Table 10. Kinetic constants of AAD-12 (v2) for three herbicide substrates

Substrate	K_m, μM (±SE)	k_{cat}, min^{-1} (±SE)	Assay method	Substrate inhibition at 2 mM
2,4-D	102 (±18.4)	54.1 (±3.1)	Phenol detection	55%
(R,S)-dichlorprop	122 (±2.7)*	63.4 (±0.5)	Phenol detection	55%
Triclopyr	241 (±30)	2.6 (±0.1)	ΔA325 nm	0%

* Because of the (S)-enantiomeric preference of AAD-12, the K_m value was calculated assuming 50% of the racemic mixture was available as a substrate

Example 7 – Transformation into *Arabidopsis* and Selection

[00202] 7.1 – *Arabidopsis thaliana* growth conditions.

[00203] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00204] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00205] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

[00206] 7.2 – *Agrobacterium* transformation.

[00207] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA)

Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

[00208] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 µl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00209] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00210] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed Agrobacterium cells, suspended in 10 µl of water, at 100° C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by

ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

[00211] 7.3 – *Arabidopsis* transformation.

[00212] *Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044µM benzylamino purine (10 µl/liter of 1 mg/ml stock in DMSO) and 300 µl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

[00213] 7.4 – Selection of transformed plants.

[00214] Freshly harvested T_1 seed [*AAD-12 (v1)* gene] was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00215] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00216] Seven days after planting (DAP) and again 11 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-12 (v1)* (plant optimized gene) to provide phenoxy auxin herbicide resistance.

[00217] T_1 plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis,* 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200g ae/ha). Tables 10 and 11 show comparisons drawn to an aryloxyalkanoate herbicide resistance gene *(AAD-1 (v3))* previously described in PCT/US2005/014737.

[00218] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

[00219] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluroxypyr, could be effectively degraded *in planta*. Herbicides

were applied to T_1 plants with use of a track sparyer in a 187 L/ha spray volume. T_1 plants that exhibited tolerance to 2,4-D DMA were further accessed in the T_2 generation.

[00220] 7.5 – Results of selection of transformed plants.

[00221] The first *Arabidopsis* transformations were conducted using *AAD-12 (v1)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T_1 seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT + Liberty are used for selection. T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 11 compares the response of *AAD-12 (v1)* and control genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The *AAD-12 (v1)* gene imparted herbicide resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 11 simply to demonstrate the significant difference between the plants transformed with *AAD-12 (v1)* versus the wildtype or *PAT/Cry1F*-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or 6x field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

Table 11. *AAD-12 (v1)* transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence, compared to an *AAD-1 v3* (T_4) homozygous resistant population, or a *Pat-Cry1F* transformed, auxin-sensitive control.

AAD-12 (v1) gene T_1 transformants Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control-buffer	6	0	0	0	0
50 g ae/ha 2,4-D	6	0	2	16	24
200 g ae/ha 2,4-D	6	1	1	11	18
800 g ae/ha 2,4-D	5	2	1	15	20
3200 g ae/ha 2,4-D	8	0	0	6	6
PAT/Cry1F (transformed control) Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	4	1	5	31	16
200 g ae/ha 2,4-D	0	0	10	70	2
800 g ae/ha 2,4-D	0	0	10	81	8
3200 g ae/ha 2,4-D	0	0	10	91	2
Homozygous AAD-1 (v3) gene T_4 plants Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	10	0	0	0	0
200 g ae/ha 2,4-D	10	0	0	0	0
800 g ae/ha 2,4-D	10	0	0	0	0
3200g ae/ha 2,4-D	9	1	0	2	6

[00222] Table 12 shows a similarly conducted dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. The data shows that the herbicidally active (R-) isomer of dichlorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-dichlorprop well enough to impart commercially acceptable tolerance is one distinguishing characteristic that separates the two genes. (Table 12). AAD-1 and AAD-12 are considered R- and S-specific α-ketoglutarate dioxygenases, respectively.

Table 12. T_1 *Arabidopsis* response to a range of R-dichlorprop rates applied postemergence.

AAD-12 v1 gene Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
Untreated control	6	0	0	0	0
50 g ae/ha R-dichlorprop	0	0	8	63	7
200 g ae/ha R-dichlorprop	0	0	8	85	10
800 g ae/ha R-dichlorprop	0	0	8	96	4
3200 g ae/ha R-dichlorprop	0	0	8	98	2

PAT/Cry1F Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	0	10	0	27	2
200 g ae/ha R-dichlorprop	0	0	10	69	3
800 g ae/ha R-dichlorprop	0	0	10	83	6
3200 g ae/ha R-dichlorprop	0	0	10	90	2

Homozygous AAD-1 (v3) gene T_4 plants	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	10	0	0	0	0
50g ae/ha R-dichlorprop	10	0	0	0	0
200 g ae/ha R-dichlorprop	10	0	0	0	0
800 g ae/ha R-dichlorprop	10	0	0	0	0
3200 g ae/ha R-dichlorprop	10	0	0	0	0

[00223] 7.6 – *AAD-12 (v1)* as a selectable marker.

[00224] The ability to use *AAD-12 (v1)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed as described above. Approximately 50 T_4 generation *Arabidopsis* seed (homozygous for *A AD-12 (v1)*) were spiked into approximately 5,000 wildtype (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 follwed by 11 DAP. Since all individuals also contained the *PAT* gene in the same transformation vector, *AAD-12* selected with 2,4-D could be directly compared to *PAT* selected with glufosinate.

[00225] Treatments were applied with a DeVilbiss spray tip as previously describd. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate *AAD-12 (v1)* can be effectively used as an alternative selectable marker for a population of transformed *Arabidopsis*.

[00226] 7.7 – Heritability.

[00227] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). Seventy-five percent of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00228] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show *AAD-12 (v1)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

[00229] 7.8 – Additional foliar applications herbicide resistance in AAD-12 *Arabidopsis*.

[00230] The ability of *AAD-12 (v1)* to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates. T_2 generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing *PAT* and the insect resistance gene *Cry1F* was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Garlon 3A, Dow AgroSciences) and 280-2240 g ae/ha fluroxypyr (Starane, Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ae/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

[00231] There is no effect of the 2,4-D metabolite, 2,4-dichlorophenol (DCP), on transgenic non-*AAD-12* control *Arabidopsis (Pat/Cry1F)*. *AAD-12*-transformed plants were also clearly protected from the triclopyr and fluroxypyr herbicide injury that was seen in the transformed non-resistant conrols (see Table 13). These results confirm that *AAD-12 (v1)* in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. This is the first report of an enzyme with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

Table 13. Comparison of T_2 AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

	Pyridyloxyacetic auxins	
	Ave % Injury 14DAT	
Herbicide Treatment	**Segregating T_2 *AAD-12 (v1)* plants (pDAB724.01.120)**	**Pat/Cry1f - Control**
280 g ae/ha Triclopyr	0	52
560 g ae/ha Triclopyr	3	58
1120 g ae/ha Triclopyr	0	75*

Table 13. Comparison of T_2 AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.		
2240 g ae/ha Triclopyr	3	75*
280 g ae/ha Fluroxypyr	0	75*
560 g ae/ha Fluroxypyr	2	75*
1120 g ae/ha Fluroxypyr	3	75*
2240 g ae/ha Fluroxypyr	5	75*
	Inactive DCP metabolite	
280 g ae/ha 2,4-DCP	0	0
560 g ae/ha 2,4-DCP	0	0
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

* Plants in this experiment were stunted and severely epinastic, but remained green and did not receive injury ratings > 75%.

[00232] 7.9 – Molecular analysis of *AAD-12 (v1) Arabidopsis*.

[00233] Invader Assay (methods of Third Wave Agbio Kit Procedures) for *PAT* gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-12 (v1)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-12 (v1)*. Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00234] Results showed that all 2,4-D resistant plants assayed, contained *PAT* (and thus by inference, *AAD-12 (v1)*). Copy number analysis showed total inserts ranged from 1 to 5 copies. This correlates, too, with the *AAD-12 (v1)* protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

[00235] 7.10 – *Arabidopsis* transformed with molecular stack of *AAD-12 (v1)* and a glyphosate resistance gene.

[00236] T_1 *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (*AAD-12 (v1)* + *EPSPS*) which encodes a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-12 (v1)* as the selectable marker as described in example 7.6. T_1 plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-12 (v1)* + *PAT* T_4 homozygous lines (pDAB724-transformed), and *PAT* + *Cry1F* homozygous

line (transformed control). The pDAB3759 and pDAB724 transformed plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

[00237] Initial resistance assessment indicated plants tolerant to 2,4-D were subsequently tolerant to glyphosate when compared to the response of the three control lines. These results indicate that resistance can be imparted to plants to two herbicides with differening modes of action, including 2,4-D and glyphosate tolerance, allowing application of both herbicides postemergence. Additionally, AAD-12 + 2,4-D was used effectively as a selectable marker for a true resistance selection.

Table 14. T_1 *Arabidopsis* response to a range of glyphosate rates applied postemergence (14 DAT).

AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	13	2	1	11	16
105 g ae/ha glyphosate	10	1	5	34	38
420 g ae/ha glyphosate	5	6	5	44	37
1680 g ae/ha glyphosate	0	0	16	85	9

PAT/Cry1F Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	67	7
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

Wildtype (Col-0) Averages	% Injury <20%	% Injury 20-40%	% Injury >40%	% Injury Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	75	13
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

pDAB724 T_4 (PAT + AAD-12) Averages	% Injury <20%	20-40%	% Injury >40%	Ave	Std Dev
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	66	8
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

[00238] 7.11 – *AAD-12 Arabidopsis* genetically stacked with *AAD-1* to give wider spectrum of herbicide tolerance.

[00239] *AAD-12 (v1)* (pDAB724) and *AAD-1 (v3)* (pDAB721) plants were reciprocally crossed and F_1 seed was collected. Eight F_1 seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F_1 plants and subjected to Western analysis to confirm the presence of both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

[00240] One hundred F_2 seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F_2 plants survived glufosinate selection fitting an expected segregation ration for two independently assorting loci for glufosinate resistance (15 R : 1S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop + 560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are consistent with an expected segregation pattern (9R: 6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlroprop or triclopyr). The results indicate that *AAD-12* (pDAB724) can be successfully stacked with *AAD-1* (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D + R-dichlorprop) and (2,4-D + fluroxypyr + triclopyr), respectively]. This could be useful to bring 2,4-D tolerance to a very sensitive species through conventional stacking of two separate 2,4-D resistance genes. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F_1 generation through paired sprays with herbicides that are exclusive between the AAD-1 and

AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively, above).

[00241] Other AAD stacks are also within the scope of the subject invention. The TfdA protein discussed elsewhere herein (Streber *et al.*), for example, can be used together with the subject AAD-12 genes to impart nover spectrums of herbicide resistance in transgenic plants of the subject invention.

Example 8 –WHISKERS-mediated transformation of corn using imazethapyr selection

[00242] 8.1 – Cloning of *AAD-12 (v1)*.

[00243] The *AAD-12 (v1)* gene was cut out of the intermediate vector pDAB3283 as an NcoI/SacI fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1 : *AAD-12 (v1)* cassette. This construct was digested with NotI and PvuI to liberate the gene cassette and digest the unwanted backbone. This was ligated to NotI cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101 or pDAS1863, and contains ZmUbi1/*AAD-12 (v1)*/ZmPer5::OsAct1/AHAS /LZmLip.

[00244] 8.2 – Callus/suspension initiation.

[00245] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00246] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was

selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00247] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

[00248] 8.3 – Cryopreservation and thawing of suspensions.

[00249] Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

[00250] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4)

in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 ml H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

[00251] 8.4 – Stable Transformation

[00252] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker shaken at 125 RPM in the dark for 30-35 minutes at 28°C, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume8.1 ml of GN6 S/M liquid medium to ~405 mg of pre-autoclaved,sterile silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00253] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml wasthen added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker at 125 RPM for 2 hours at 28°C before being filtered onto

Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00254] Approximately 2 ml of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box.

[00255] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 3 μM imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8). Plates were placed in boxes and cultured for an additional week.

[00256] Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 ml of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121°C) containing 3 μM imazethapyr from Pursuit® DG. The tissue was broken up and the 3 ml of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured until putative isolates appeared.

[00257] 8.4.1- Protocol for Isolate recovery and regeneration.

[00258] Putatively transformed events were isolated off the Pursuit®-containing embedded plates approximately 9 weeks post-transformation by transferring to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth was evident after approximately 2-3 weeks, the event was deemed to be resistant and was submitted for molecular analysis.

[00259] Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3 μM imazethapyr from Pursuit® DG, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week, then higher light (40 $\mu Em^{-2}s^{-1}$) for another week, before being transferred to regeneration medium, 36 (3P), which was identical to 28 (3P) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L

Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

[00260] From 4 experiments, full plantlets, comprised of a shoot and root, were formed *in vitro* on the embedded selection plates under dark conditions without undergoing a traditional callus phase. Leaf tissue from nine of these "early regenerators" were submitted for coding region PCR and Plant Transcription Unit (PTU) PCR for the *AAD-12* gene and gene cassette, respectively. All had an intact *AAD-12* coding region, while 3 did not have a full-length PTU (Table 15). These "early regenerators" were identified as 4101 events to differentiate them from the traditionally-derived events, which were identified as "1283" events.Plants from 19 additional events, obtained via standard selection and regeneration, were sent to the greenhouse, grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T_1 seed. Some of the events appear to be clones of one another due to similar banding patterns following Southern blot, so only 14 unique events were represented. T_0 plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (*AHAS* gene) indicated instertion complexity ranging from 1 to >10 copies. Thirteen events contained the compete coding region for *AAD-12*; however, further anaylsis indicated the complete plant transformation unit had not been incorporated for nine events. None of the compromised 1863 events were advanced beyond the T1 stage and further characterization utilized the 4101 events.

[00261] 8.5– Molecular Analysis: Maize Materials and Methods.

[00262] 8.5.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00263] 8.5.2 – Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl is placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well is overlaid with 15 µl

of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

[00264] 8.5.3 – Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-12 (v1)* PTU are Forward – GAACAGTTAGACATGGTCTAAAGG (SEQ ID NO:8) and Reverse – GCTGCAACACTGATAAATGCCAACTGG (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes.

[00265] Primers for *AAD-12 (v1)* Coding Region PCR are Forward – ATGGCTCAGACCACTCTCCAAA (SEQ ID NO:10) and Reverse – AGCTGCATCCATGCCAGGGA (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00266] 8.5.4 – Southern blot analysis.

[00267] Southern blot analysis is performed with genomic DNA obtained from Qiagen DNeasy kit. A total of 2 μg of genomic leaf DNA or 10 μg of genomic callus DNA is subjected to an overnight digestion using BSM I and SWA I restriction enzymes to obtain PTU data .

[00268] After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85 % agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100

microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60°C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

[00269] 8.6 – Postemergence herbicide tolerance in AAD-12 transformed T_0 Corn.

[00270] Four T_0 events were allowed to acclimate in the greenhouse and were grown until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of either Pursuit® (imazethapyr) or 2,4-D Amine 4. Pursuit® was sprayed to demonstrate the function of the selectable marker gene present within the events tested. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. Plants were sprayed with either a lethal dose of imazethapyr (70 g ae/ha) or a rate of 2,4-D DMA salt capable of significant injury to untransformed corn lines (2240 g ae/ha). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

[00271] Several individuals were safened from the herbicides to which the respective genes were to provide resistance. The individual clone '001' from event "001" (a.k.a.,4101(0)-001-001), however, did incur minor injury but recovered by 14 DAT. Three of the four events were moved forward and individuals were crossed with 5XH751 and taken to the next generation. Each herbicide tolerant plant was positive for the presence of the *AAD-12* coding region (PCR assay) or the presence of the *AHAS* gene (Invader assay) for 2,4-D and imazethapyr-tolerant plants, respectively. AAD-12 protein was detected in all 2,4-D tolerant T_0 plants events containing an intact coding region. The copy number of the transgene(s)

(*AHAS*, and by inference *AAD-12*) varied significantly from 1 to 15 copies. Individual T_0 plants were grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T_1 seed.

Table 15. Characterization of T_0 corn plants transformed with *AAD-12*.

Event	Spray Treatment	% Injury (14 DAT)	AAD-12 ELISA (ppm TSP)	AAD12 PCR (Coding Region)	AAD12 PCR (PTU)	AHAS Copy # (Invader)
4101(0)003. 001	2240 g ae/ha 2,4-D	0	146.9	+	+	1
4101(0)003.003	2240 g ae/ha 2,4-D	0	153.5	+	+	1
4101(0)005.001	2240 g ae/ha 2,4-D	0	539.7	+	+	9
4101(0)005.0012	0 g ae/ha 2,4-D	0	562.9	+	+	7
4101(0)001.001	70 g ae/ha imazethapyr	5	170.7	+	+	6
4101(0)002.001	0 g ae/ha imazethapyr	0	105.6	+	-	2
4101(0)002.002	70 g ae/ha imazethapyr	0	105.3	+	-	2
4101(0)003.002	70 g ae/ha imazethapyr	0	0	+	band smaller than expected	15

[00272] 8.7 – Verification of high 2,4-D tolerance in T_1 corn.

[00273] T_1 *AAD-12 (v1)* seed were planted into 3-inch pots containing Metro Mix media and at 2 leaf stage were sprayed with 70 g ae/ha imazethapyr to eliminate nulls. Surviving plants were transplanted to 1-gallon pots containing Metro Mix media and placed in the same growth conditions as before. At V3-V4 stage the plants were sprayed in the track sprayer set to 187 L/ha at either 560 or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to 5XH751 x Hi II control plants. A grading scale of 0-10 (no injury to extreme auxin injury) was developed to distinguish brace root injury. Brace Root grades were taken on 14DAT to show 2,4-D tolerance. 2,4-D causes brace root malformation, and is a consistent indicator of auxinic herbicide injury in corn. Brace root data (as seen in the table below) demonstrates that 2 of the 3 events tested were robustly tolerant to 2240 g ae/ha 2,4-D DMA. Event "pDAB4101(0)001.001" was apparently unstable; however, the other two events were robustly tolerant to 2,4-D and 2,4-D + imazethapyr or 2,-4D + glyphosate (see Table 16).

Table 16. Brace Root injury of *AAD-12 (v1)* transformed T_1 plants and Untransformed control corn plants. A scale of 0-10, 10 being the highest, was used for grading the 2,4-D DMA injury. Results are a visual average of four replications per treatment.

Herbicide	Untransformed Control	*AAD-12 (v1)* pDAB4101(0)003.003	*AAD-12 (v1)*pDAB4101(0)001.001	*AAD-12 (v1)* pDAB4101(0)005.001
	Average Brace Root Injury (0-10 Scale)			
0 g ae/ha 2,4-D DMA	0	0	0	0
2240 g ae/ha 2,4-D DMA	9	1	8	0

[00274] 8.8 – *AAD-12 (v1)* heritability in corn.

[00275] A progeny test was also conducted on seven *AAD-12 (v1)* T_1 families that had been crossed with 5XH751. The seeds were planted in three-inch pots as described above. At the 3 leaf stage all plants were sprayed with 70 g ae/ha imazethapyr in the track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Four out of the six lines tested segregated as a single locus, dominant Mendelian trait (1R:1S) as determined by Chi square analysis. Surviving plants were subsequently sprayed with 2,4-D and all plants were deemed tolerant to 2,4-D (rates ≥ 560 g ae/ha). AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species when reciprocally crossed to a commercial hybrid.

[00276] 8.9 – Stacking of *AAD-12 (v1)* to increase herbicide spectrum

[00277] *AAD-12 (v1)* (pDAB4101) and and elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F_1 seed was collected. The seed from two F_1 lines were planted and treated with 70 g ae/ha imazethapyr at the V2 stage to eliminate nulls. To the surviving plants, reps were separated and either treated with 1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr (to confirm presence of AHAS gene) or 1120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate (to confirm the presence of the Round Up Ready gene) in a track sprayer calibrated to 187 L/ha. Plants were graded 3 and 16 DAT. Spray data showed that *AAD-12 (v1)* can be conventionally stacked with a glyphosate tolerance gene (such as the Roundup CP4-EPSPS gene) or other herbicide tolerance genes to provide an increased spectrum of herbicides that may be applied safely to corn. Likewise imidazolinone + 2,4-D +glyphosate

tolerance was observed in F_1 plants and showed no negative phenotype by the molecular or breeding stack combinations of these multiple transgenes.

Table 17. Data demonstrating increase herbicide tolerance spectrum resulting from an F_1 stack of *AAD-12 (v1)* and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).

Herbicide	**Untransformed Control**	**2P782 (*Roundup Ready* Control)**	***AAD-12 (v1)* pDAB4101(0) 003.R003.AF**	***AAD-12 (v1)* pDAB4101(0) 005.R001.AF**
	Average % Injury 16DAT			
0 g ae/ha 2,4-D DMA	0	0	0	0
1120 g ae/ha 2,4-D DMA	21	19	0	0
1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr	100	100	5	1
01120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate	100	71	2	5

[00278] 8.10- Field tolerance of pDAB4101 transformed corn plants to 2,4-D, triclopyr and fluroxypyr herbicides.

[00279] Field level tolerance trials were conducted on two *AAD-12 (v1)* pDAB4101 events (4101(0)003.R.003.AF and 4101(0)005.R001.AF) and one Roundup Ready (RR) contol hybrid (2P782) at Fowler, Indiana and Wayside, Mississippi. Seeds were planted with cone planter on 40-inch row spacing at Wayside and 30 inch spacing at Fowler. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. The *AAD-12 (v1)* events contained the *AHAS* gene as a selectable marker. The F_2 corn events were segregating so the *AAD-12 (v1)* plants were treated with imazethapyr at 70 g ae/ha to remove the null plants. Herbicide treatments were applied when corn reached the V6 stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after treatment. Brace root injury ratings were taken at 28DAT on a scale of 0-10 with 0-1 being slight brace root fusing, 1-3 being moderate brace root swelling/wandering and root proliferation, 3-5 being moderate brace root fusing, 5-9 severe brace root fusing and malformation and 10 being total inhibition of brace roots.

[00280] *AAD-12 (v1)* event response to 2,4-D, triclopyr, and fluroxypyr at 14 days after treatment are shown in Table 18. Crop injury was most severe at 14 DAT. The RR control

corn (2P782) was severely injured (44% at 14 DAT) by 2,4-D at 4480 g ae/ha, which is 8 times (8X) the fnormal field use rate. The *AAD-12 (v1)* events all demonstrated excellent tolerance to 2,4-D at 14 DAT with 0% injury at the 1, 2 and 4X rates, respectively. The control corn (2P782) was severely injured (31% at 14 DAT) by the 2X rate of triclopyr (840 g ae/ha). *AAD-12 (v1)* events demonstrated tolerance at 2X rates of triclopyr with an average of 3% injury at 14 DAT across the two events. Fluroxypyr at 280 g ae/ha caused 11% visual injury to the wild-type corn at 14 DAT. *AAD-12 (v1)* events demonstrated increased tolerance with an average of 8% injury at 5 DAT.

[00281] Applications of auxinic herbicides to corn in the V6 growth stage can cause malformation of the brace roots. Table 18 shows the severity of the brace root injury caused by 2,4-D, triclopyr, and fluroxypyr. Triclopyr at 840 g ae/ha caused the most severe brace root fusing and malformation resulting in an average brace root injury score of 7 in the 2P782 control-type corn. Both *AAD-12 (v1)* corn events showed no brace root injury from the triclopyr treatment. Brace root injury in 2P782 corn increased with increasing rates of 2,4-D. At 4480 g ae/ha of 2,4-D, the *AAD-12* events showed no brace root injury; whereas, severe brace root fusing and malformation was seen in the 2P782 hybrid. Fluroxypyr caused only moderate brace root swelling and wandering in the wild-type corn with the *AAD-12 (v1)* events showing no brace root injury.

[00282] This data clearly shows that *AAD-12(v1)* conveys high level tolerance in corn to 2,4-D, triclopyr and fluroxypyr at rates far exceeding those commercially used and that cause non-*AAD-12 (v1)* corn severe visual and brace root injury.

Table 18. Visual Injury of AAD-12 events and wild-type corn following foliar applications of 2,4-D, triclopyr and fluroxypyr under field conditions.

		%Visual Injury 14DAT		
Treatment	Rate (g ae/ha)	AAD-12 4101(0)003.R.003.AF	AAD-124101(0)005.001.AF	2P782 control
Untreated	0	0	0	0
2,4-D	1120	0	0	9
2,4-D	2240	0	1	20
2,4-D	4480	0	1	34
Fluroxypyr	280	1	5	11
Triclopyr	840	3	4	31
Dicamba	840	8	8	11

Table 19. Brace root injury ratings for AAD-12 and wild-type corn plants in response to 2,4-D, triclopyr and fluroxypyr under field conditions.

		Brace Root Injury Rating (0-10 scale) 28DAT		
Treatment	Rate (g ae/ha)	AAD-12 event 4101(0)003.R.003.AF	AAD-12 event 4101(0)005.001.AF	Wild-type NK603
Untreated	0	0	0	0
2,4-D	1120	0	0	3
2,4-D	2240	0	0	5
2,4-D	4480	0	0	6
Fluroxypyr	280	0	0	2
Triclopyr	840	0	0	7
Dicamba	840	1	1	1

Example 9. Protein detection from transformed plants via antibody

[00283] 9.1 – Extracting AAD-12 (v1) from plant leaves.

Approximately 50 to 100 mg of leaf tissue was cut into small pieces (or 4 single-hole-punched leaf discs) and put into 2-ml cluster tubes containing 2 stainless steel BB beads (4.5 mm; Daisy Co., cat. #145462-000). Five hundred microliters of plant extraction buffer (PBS containing 0.05 % Tween 20 and 1% Bovine serum albumin) was added to each sample. The tubes were capped and secured in the Geno/Grinder (Model 2000-115, Certiprep, Metuchen, NJ) and shaken for 6 min with setting at 1x of 500 rpm. Tubes were centrifuged at 5000x g for 10 min and supernatant containing the soluble proteins were analyzed for AAD-12 (v1) using Western Blots and ELISA.

[00284] 9.2 – Enzyme linked immuno-sorbent assay (ELISA).

[00285] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-12 antibody (0.5 µg/ml) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 µL standard AAD-12 of known concentrations or plant extracts from different samples were incubated in the wells. For standard curve, purified AAD-12 was diluted 2-fold serially from 52 to 0.813 ng/ml in triplicates. Plant extracts were diluted 5, 10, 20, and 40-fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-12 antibody-HRP conjugate (0.5 ug/ml) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-

Step™ Ultra TMB-ELISA (Pierce, Rockford, IL), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 µL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-12 (v1) in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[00286] For comparison, each sample was normalized with its fresh weight and percent expression was calculated.

[00287] 9.3 – Western blotting analysis.

[00288] Plant extracts or AAD-12 standards (5 and 0.5 µg/ml) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-12 (v1) protein was detected by anti-AAD-12 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham, NJ).

Example 10 – Tobacco Transformation

[00289] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. KY160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB3278, aka pDAS1580, *AAD-12 (v1) + PAT*), grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing

250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30°C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

[00290] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[00291] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-12 (v1)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00292] The DNA samples were diluted to 9 ng/µl and then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the %

signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

[00293] All events were also assayed for the presence of the *AAD-12 (v1)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR *AAD-12* were (SdpacodF: ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:12) and (SdpacodR: CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:13). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably *AAD-12 (v1)* since these genes are physically linked) were regenerated and moved to the greenhouse.

[00294] 10.1 Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T_0 tobacco

[00295] T_0 plants from each of the 19 events were challenged with a wide range of 2,4-D, triclopyr, or fluroxypyr sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluroxypyr was likewise applied at 35, 140, or 560 g ae/ha. Triclopyr was applied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ae/ha (equivalent to 4X field rate). On the whole, *AAD-12 (v1)* events were more sensitive to fluroxypyr, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T_0 plant responses to 560 g ae/ha fluroxypyr. Events were categorized into "low" (>40% injury 14 DAT), "medium" (20-40% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable."

Table 20. Tobacco T0 events transformed with pDAS1580 (*AAD-12 (v1) + PAT*)

# Tube	Plant ID	Copy # *PAT*	PTU PCR *AAD-12*	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance[@]
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+	*	*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable
24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High

[@] Distinguishing herbicide tolerance performance of events required assessment of relative tolerance when treated with 560 g ae/ha fluroxypyr where tolerance was variable across events.

[00296] 10.2 Verification of high 2,4-D tolerance in T_1 tobacco.

[00297] Two to four T_0 individuals surviving high rates of 2,4-D and fluroxypyr were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 7.4), followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (*PAT* gene selection). Survivors were transferred to individual 3-

inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY160 tobacco. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 140 - 2240 g ae/ha 2,4-D dimethylamine salt (DMA), 70-1120 g ae/ha triclopyr or 35-560 g ae/ha fluroxypyr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity.

[00298] No injury was observed at 4X field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line, but the greatest injury was observed with fluroxypyr. The fluroxypyr injury was short-lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT (Table 21). It is important to note that untransformed tobacco is exceedingly sensitive to fluroxypyr. These results indicated commercial level 2,4-D tolerance can be provided by *AAD-12 (v1)*, even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluroxypyr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

Table 21. Assessment of cross tolerance of *AAD-12 (v1)* T_1 tobacco plants' response to various phenoxy and pyridyloxy auxin herbicides.

Herbicide	KY160 – Wildtype	1580(1)-004 (high tolerance in T_0 generation)	1580(1)-018 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
140 g ae/ha 2,4-D DMA	45	0	0
560 g ae/ha 2,4-D DMA	60	0	0
2240 g ae/ha 2,4-D DMA	73	0	0
70 g ae/ha triclopyr	40	0	5
280 g ae/ha triclopyr	65	0	5
1120 g ae/ha triclopyr	80	0	8
35 g ae/ha fluroxypyr	85	0	8
140 g ae/ha fluroxypyr	93	0	10
560 g ae/ha fluroxypyr	100	3	18

[00299] 10.3 *AAD-12 (v1)* heritability in tobacco

[00300] A 100 plant progeny test was also conducted on seven T_1 lines of *AAD-12 (v1)* lines. The seeds were stratified, sown, and transplanted with respect to the procedure above with the exception that null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

10.4 – Field tolerance of pDAS1580 tobacco plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

[00301] Field level tolerance trials were conducted on three *AAD-12 (v1)* lines (events pDAS1580-[1]-018.001, pDAS1580-[1]-004.001 and pDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Mississippi. Tobacco transplants were grown in the greenhouse by planting T_1 seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

[00302] The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after treatment.

[00303] *AAD-12 (v1)* event response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 22. The non-transformed tobacco line was severely injured (63% at 14 DAT) by 2,4-D at 560 g ae/ha which is considered the 1X field application rate. The *AAD-12 (v1)* lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8X rates, respectively. The non-transformed tobacco line was severely injured (53% at 14 DAT) by the 2X rate of triclopyr (840 g ae/ha); whereas, *AAD-*

12 (v1) lines demonstrated tolerance with an average of 5% injury at 14 DAT across the three lines. Fluroxypyr at 280 g ae/ha caused severe injury (99%) to the non-transformed line at 14 DAT. *AAD-12 (v1)* lines demonstrated increased tolerance with an average of 11% injury at 14 DAT.

[00304] These results indicate that *AAD-12 (v1)* transformed event lines displayed a high level of tolerance to 2,4-D, triclopyr and fluroxypyr at multiples of commercial use rates that were lethal or caused severe epinastic malformations to non-transformed tobacco under representative field conditions.

Table 22. *AAD-12 (v1)* tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

Herbicide Treatment		Average % Injury across locations at 14 DAT			
Active Ingredient	Rate	Wild type	pDAS1580-[1]-004.001	pDAS1580-[1]-020.016	pDAS1580-[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxypyr	280 GM AE/HA	99	11	11	12

[00305] 10.5 *AAD-12 (v1)* protection against elevated 2,4-D rates

[00306] Results showing *AAD-12 (v1)* protection against elevated rates of 2,4-D DMA in the greenhouse are shown in Table 23. T_1 *AAD-12 (v1)* plants from an event segregating 3R:1S when selected with 560 g ai/ha Liberty using the same protocol as previously described. T_1 *AAD-1 (v3)* seed was also planted for transformed tobacco controls (*see* PCT/US2005/014737). Untransformed KY160 was served as the sensitive control. Plants were sprayed using a track sprayer set to 187 L/ha at 140, 560, 2240, 8960, and 35840 g ae/ha 2,4-D DMA and rated 3 and 14 DAT.

[00307] *AAD-12 (v1)* and *AAD-1 (v3)* both effectively protected tobacco against 2,4-D injury at doses up to 4X commercial use rates. *AAD-12 (v1),* however, clearly demonstrated a marked advantage over *AAD-1 (v3)* by protecting up to 64X the standard field rates.

Table 23. Results demonstrating protection provided by *AAD-12 (v1)* and *AAD-1 (v3)* against elevated rates of 2,4-D.

	KY160 control	*AAD-1(v3)*	*AAD-12 (v1)*
Treatment	Average % injury 14 DAT		
2240 g ae/ha 2,4-D	95	4	0
8960 g ae/ha 2,4-D	99	9	0
35840 g ae/ha 2,4-D	100	32	4

[00308] 10.6 Stacking of *AAD-12* to increase herbicide spectrum

[00309] Homozygous *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) plants (*see* PCT/US2005/014737 for the latter) were both reciprocally crossed and F_1 seed was collected. The F_1 seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regimine as used for the other testing with one of the following treatments: 70, 140, 280 g ae/ha fluroxypyr (selective for the *AAD-12 (v1)* gene); 280, 560, 1120 g ae/ha R-dichloroprop (selective for the *AAD-1 (v3)* gene); or 560, 1120, 2240 g ae/ha 2,4-D DMA (to confirm 2,4-D tolerance). Homozygous T_2 plants of each gene were also planted for use as controls. Plants were graded at 3 and 14 DAT. Spray results are shown in Table 24.

[00310] The results confirm that *AAD-12 (v1)* can be successfully stacked with *AAD-1 (v3)*, thus increasing the spectrum herbicides that may be applied to the crop of interest (phenoxyactetic acids + phenoxypropionic acids vs penoxyacetic acids + pyridyloxyacetic acids for AAD-1 and AAD-12, respectively). The complementary nature of herbicide cross resistance patterns allows convenient use of these two genes as complementary and stackable field-selectable markers. In crops where tolerance with a single gene may be marginal, one skilled in the art recognizes that one can increase tolerance by stackinga second tolerance gene for the same herbicide. Such can be done using htesame gene with the same or different promoters; however, as observed here, stacking and tracking two completmentary traits can be facilitated by the distinguishing cross protection to phenoxypropionic acids [from *AAD-1 (v3)*] or pyidyloxyacetic acids [*AAD-12 (v1)*]

Table 24. Comparison of auxinic herbicide cross tolerance of *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) T_2 plants compared to AAD-12 x AAD-1 F1 cross and to wildtype.

Treatment	KY160 wildtype control	*AAD-12 (v1)* (pDAS1580)	*AAD-1(v3)* (pDAB721)	*AAD-12 (v1)* x *AAD (v3)* F_1
	Average % injury 14 DAT			
560 g ae/ha 2,4-D	63	0	0	0
1120 g ae/ha 2,4-D	80	0	4	0
2240 g ae/ha 2,4-D	90	0	9	0
280 g ae/ha R-dichlorprop	25	15	0	0
560 g ae/ha R-dichlorprop	60	50	0	0
1120 g ae/ha R-dichlorprop	80	70	3	0
70 g ae/ha fluroxypyr	40	0	40	0
140 g ae/ha fluroxypyr	65	0	60	0
280 g ae/ha fluroxypyr	75	3	75	3

Example 11 - Soybean Transformation

[00311] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00312] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean,

zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-12 (v1)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-12 (v1)*.

[00313] 11.1 Gateway cloning of binary constructs

[00314] The *AAD-12 (v1)* coding sequence was cloned into five different Gateway Donor vectors containing different plant promoters. The resulting *AAD-12 (v1)* plant expression cassettes were subsequently cloned into a Gateway Destination Binary vector via the LR Clonase reaction (Invitrogen Corporation, Carlsbad Ca, Cat # 11791-019).

[00315] An NcoI – SacI fragment containing the *AAD-12 (v1)* coding sequence was digested from DASPICO12 and ligated into corresponding NcoI – SacI restriction sites within the following Gateway Donor vectors: pDAB3912 (attL1 // CsVMV promoter // AtuORF23 3'UTR // attL2); pDAB3916 (attL1 // AtUbi10 promoter // AtuORF23 3'UTR // attL2); pDAB4458 (attL1 // AtUbi3 promoter // AtuORF23 3'UTR // attL2); pDAB4459 (attL1 // ZmUbi1 promoter // AtuORF23 3'UTR // attL2); and pDAB4460 (attL1 // AtAct2 promoter // AtuORF23 3'UTR // attL2). The resulting constructs containing the following plant expression cassettes were designated: pDAB4463 (attL1 // CsVMV promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2); pDAB4467 (attL1 // AtUbi10 promoter // AAD-12 (v1) // AtuORF23 3'UTR // attL2); pDAB4471 (attL1 // AtUbi3 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2); pDAB4475 (attL1 // ZmUbi1 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2); and pDAB4479 (attL1 // AtAct2 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // attL2). These constructs were confirmed via restriction enzyme digestion and sequencing.

[00316] The plant expression cassettes were recombined into the Gateway Destination Binary vector pDAB4484 (RB7 MARv3 // attR1 – ccdB – chloramphenicol resistance – attR2 //

CsVMV promoter // PATv6 // AtuORF1 3'UTR) via the Gateway LR Clonase reaction. Gateway Technology uses lambda phage-based site-specific recombination instead of restriction endonuclease and ligase to insert a gene of interest into an expression vector. Invitrogen Corporation, Gateway Technology: A Universal Technology to Clone DNA Sequences for Functional Analysis and Expression in multiple Systems, Technical Manual, Catalog #'s 12535-019 and 12535-027, Gateway Technology Version E, 9/22/2003, #25-022. The DNA recombination sequences (attL, and attR,) and the LR Clonase enzyme mixture allows any DNA fragment flanked by a recombination site to be transferred into any vector containing a corresponding site. The attL1 site of the donor vector corresponds with attR1 of the binary vector. Likewise, the attL2 site of the donor vector corresponds with attR2 of the binary vector. Using the Gateway Technology the plant expression cassette (from the donor vector) which is flanked by the attL sites can be recombined into the attR sites of the binary vector. The resulting constructs containing the following plant expression cassettes were labeled as: pDAB4464 (RB7 MARv3 // CsVMV promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR); pDAB4468 (RB7 MARv3 // AtUbi10 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR); pDAB4472 (RB7 MARv3 // AtUbi3 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter// *PATv6* // AtuORF1 3'UTR); pDAB4476 (RB7 MARv3 // ZmUbi1 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR); and pDAB4480 (RB7 MARv3 // AtAct2 promoter // *AAD-12 (v1)* // AtuORF23 3'UTR // CsVMV promoter // *PATv6* // AtuORF1 3'UTR) (see Table 8). These constructs were confirmed via restriction enzyme digestion and sequencing.

[00317] 11.2 Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00318] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin

followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00319] 11.2.1 – Plant transformation production of *AAD-12 (v1)* tolerant phenotypes. Seed derived explants of "Maverick"and the *Agrobacterium* mediated cot-node transformation protocol was used to producw *AAD-12 (v1)* transgenic plants.

[00320] 11.2.2– *Agrobacterium* preparation and inoculation

[00321] *Agrobacterium* strain EHA101 (Hood et al. 1986), carrying each of five binary pDAB vectors (Table 8) was used to initiate transformation. Each binary vector contains the *AAD-12 (v1)* gene and a plant-selectable gene (*PAT*) cassette within the T-DNA region. Each gene is driven by the promoters listed in Table 8 and these plasmids were mobilized into the EHA101 strain of *Agrobacterium* by electroporation. The selected colonies were then analyzed for the integration of genes before the Agrobacterium treatment of the soybean explants. Maverick seeds were used in all transformation experiments and the seeds were obtained from University of Missouri, Columbia, MO.

[00322] Agrobacterium-mediated transformation of soybean (*Glycine max*) using the *PAT* gene as a selectable marker coupled with the herbicide glufosinate as a selective agent was carried out followed a modified procedure of Zeng et al. (2004). The seeds were germinated on B5 basal medium (Gamborg et al. 1968) solidified with 3 g/L Phytagel (Sigma-Aldrich, St. Louis, Mo.); added l-cysteine to the co-cultivation medium at 400 mg/L and co-cultivation

lasted 5 days (Olhoft and Somers 2001); shoot initiation, shoot elongation, and rooting media were supplemented with 50 mg/L cefotaxime, 50 mg/L timentin, 50 mg/L vancomycin, and solidified with 3 g/L Phytagel. Selected shoots were then transferred to the rooting medium. The optimal selection scheme was the use of glufosinate at 8 mg/L across the first and second shoot initiation stages in the medium and 3–4 mg/L during shoot elongation in the medium.

[00323] Prior to transferring elongated shoots (3–5 cm) to rooting medium, the excised end of the internodes were dipped in 1 mg/L indole 3-butyric acid for 1–3 min to promote rooting (Khan et al. 1994). The shoots struck roots in 25×100 mm glass culture tubes containing rooting medium and then they were transferred to soil mix for acclimatization of plantlets in Metro-mix 200 (Hummert International, Earth City, Mo.) in open Magenta boxes in Convirons. Glufosinate, the active ingredient of Liberty herbicide (Bayer Crop Science), was used for selection during shoot initiation and elongation. The rooted plantlets were acclimated in open Magenta boxes for several weeks before they were screened and transferred to the greenhouse for further acclimation and establishment.

[00324] 11.2.3 - Assay of putatively transformed plantlets, and analyses established T_0 plants in the greenhouse.

[00325] The terminal leaflets of selected leaves of these plantlets were leaf painted with 50 mg/L of glufosinate twice with a week interval to observe the results to screen for putative transformants. The screened plantlets were then transferred to the greenhouse and after acclimation the leaves were painted with glufosinate again to confirm the tolerance status of these plantlets in the GH and deemed to be putative transformants.

[00326] Plants that are transferred to the greenhouse can be assayed for the presence of an active *PAT* gene further with a non-destructive manner by painting a section of leaf of the T_0 primary transformant, or progeny thereof, with a glufosinate solution [0.05-2% v/v Liberty Herbicide, preferably 0.25-1.0% (v/v),=500-2000 ppm glufosinate, Bayer Crop Science]. Depending on the concentration used, assessment for glufosinate injury can be made 1-7 days after treatment. Plants can also be tested for 2,4-D tolerance in a non-destructive manner by selective application of a 2,4-D solution in water (0.25-1% v/v commercial 2,4-D dimethylamine salt formulation, preferably 0.5% v/v = 2280 ppm 2,4-D ae) to the terminal leaflet of the newly expanding trifoliolate one or two, preferably two, nodes below the youngest emerging trifolioate. This assay allows assessment of 2,4-D sensitive plants 6 hours to several days after application by assessment of leaf flipping or rotation >90 degrees from

the plane of the adjacent leaflets. Plants tolerant to 2,4-D will not respond to 2,4-D. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-12 (v1)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically comprise one or two selective rates in the range of 100-1120 g ae/ha using a track sprayer as previously described. T_1 plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance (in plants transformed with constructs that contain EPSPS) or another glyphosate tolerance gene can be assessed in the T_1 generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha glyphosate. Analysis of protein expression will occur as described in below. Individual T_0 plants were assessed for the presence of the coding region of the gene of interest (*AAD-12 (v1)* or *PAT v6*) and copy number. Determination of the inheritance of *AAD-12 (v1)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance as described in previous examples.

[00327] A subset of the initial transformants were assessed in the T_0 generation according to the methods above. Any plant confirmed as having the *AAD-12 (v1)* coding region, regardless of the promoter driving the gene did not respond to the 2,4-D leaf painting whereas wildtype Maverick soybeans did (Table Sec 11.2.3). *PAT*-only transformed plants responded the same at wildtype plants to leaf paint applications of 2,4-D

[00328] 2,4-D was applied to a subset of the plants that were of similar size to the wildtype control plants with either 560 or 1120 g ae 2,4-D. All *AAD-12 (v1)*-containing plants were clearly resistant to the herbicide application versus the wildtype Maverick soybeans. A slight level of injury (2 DAT) was observed for two *AAD-12 (v1)* plants, however, injury was temporary and no injury was observed 7 DAT. Wildtpe control plants were severely injured 7-14 DAT at 560 g ae/ha 2,4-D and killed at 1120 g ae/ha. These data are consistent with the fact that *AAD-12 (v1)* can impart high tolerance (>2X field rates) to a sensitive crop like soybeans. The screened plants were then sampled for molecular and biochemical analyses for the confirmation of the *AAD12 (v1)* genes integration, copy number, and their gene expression levels as described below and reported in Table 25.

Table 25. T_0 soybean response to 2,4-D leaf paint and 2,4-D spray application.

Construct (pDAB#)	Gene	Promoter	Event	NODE LEAF PAINTED	Leaf flip assay 2,4-D @ (18 HAT) Node N-1	Node N-2	Spray POST over the top with 2,4-D (g ae/ha)	Stage at appl (# nodes)	ELISA] (ng/mL)	Southern Copy number	PCR coding region	% injury 2 DAT	% injury 7 DAT	% injury 14 DAT
4464	AAD-12	CsVMV	D-1-14	N-1	0		0	>10	5246.83	2	+	X	X	0
4464	AAD-12	CsVMV	D-2-9	N-2		0	0	>10	204.27	1	+	X	X	0
4468	AAD-12	AtUbi10	D-3-7	N-2		0	0	>10	4.65	1	+	0	0	0
4468	AAD-12	AtUbi10	D-4-11B	N-2		0	0	8	1452.84	2	+	0	0	0
4468	AAD-12	AtUbi10	D-4-16	N-2		0	0	>10	653.21	2	+	X	X	0
4480	AAD-12	AtAct2	D-9-1	N-2		0	0	>10	248.33	3 or 4	+	X	X	0
4464	AAD-12	CsVMV	D-2-14	N-2		0	560	7	4917.43	2	+	0	0	0
4468	AAD-12	AtUbi10	D-3-5	N-2		0	560	8	365.75	1	+	0	0	0
4468	AAD-12	AtUbi10	D-3-6	N-1	0		560	5	714.79	3	+	0	0	0
4472	AAD-12	AtUbi3	D-5-2	N-1	0		560	6	0.58	1	+	5	0	0
4468	AAD-12	AtUbi10	D-3-9	N-2		0	1120	6	2657.26	3	+	0	0	0
4468	AAD-12	AtUbi10	D-4-17	N-2		0	1120	7	286.14	5	+	5	0	0
4499	PAT	CsVMV	D-2-3	N-2		1	0	>10	2.36	5	+	X	X	0
Maverick	WT		WT-10	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-2	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-3	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-4	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-5	NT			560	4	ND	ND	ND	50	60	60
Maverick	WT		WT-6	NT			560	4	ND	ND	ND	70	90	80
Maverick	WT		WT-7	NT			560	4	ND	ND	ND	70	80	80
Maverick	WT		WT-10	NT			1120	4	ND	ND	ND	70	90	100
Maverick	WT		WT-8	NT			1120	4	ND	ND	ND	70	95	100
Maverick	WT		WT-9	NT			1120	4	ND	ND	ND	70	95	100
					1=Flip				ND=Not determined					
					0=No Flip									

[00329] 11.2.4– Molecular Analyses: Soybean

[00330] 11.2.4.1– Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μL.

[00331] 11.2.4.2- Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-12 (v1)* PTU are (Forward – ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:8) and (Reverse – CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR AAD-12 (v1) are (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:10) and (Reverse – CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00332] 11.2.4.3- Southern blot analysis. Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 10 μg of genomic DNA is subjected to an overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85 % agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene

UV stratalinker 1800) at 1200 X100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

[00333] 11.2.5 – Biochemical Analyses: Soybean

[00334] 11.2.5.1 – Tissue Sampling and Extracting AAD-12 (v1) protein from soybean leaves. Approximately 50 to 100 mg of leaf tissue was sampled from the N-2 leaves that were 2,4-D leaf painted, but after 1 DAT. The terminal N-2 leaflet was removed and either cut into small pieces or 2-single-hole- punched leaf discs (~0.5 cm in diameter) and were frozen on dry ice instantly. Further protein analysis (ELISA and Western analysis) was completed according to methods described in Example 9.

[00335] 11.2.6 – T_1 Progeny evaluation. T_0 plants will be allowed to self fertilize to derive T_1 families. Progeny testing (segregation analysis) will be assayed using glufosinate at 560 g ai/ha as the selection agent applied at the V1-V2 growth stage. Surving plants will be further assayed for 2,4-D tolerance at one or more growth stages from V2-V6. Seed will be produced through self fertilization to allow broader herbicide testing on the transgenic soybean.

[00336] *AAD-12 (v1)* transgenic Maverick soybean plants have been generated thorugh *Agrobacterium*- mediated cot-node transformation system. The T_0 plants obtained tolerated up to 2X levels of 2,4-D field applications and developed fertile seeds. The frequency of fertile transgenic soybean plants was up to 5.9%. The integration of the *AAD1-12 (v1)* gene into the soybean genome was confirmed by Southern blot analysis. This analysis indicated that most of the transgenic plants contained a low copy number. The plants screened with

AAD-12 (v1) antibodies showed positive for ELISA and the appropriate band in Western analysis.

[00337] 11.3 Transformation Method 2: Aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00338] Culture of embryogenic soybean callus tissue and subsequent beaming can be accomplished as described in U.S. Patent No. 6,809,232 (Held *et al.*) to create transformants using one or more constructs in Table 8.

[00339] 11.4 Transformation Method 3. Biolistic bombardment of Soybean

[00340] This can be accomplished using mature seed derived embryonic axes meristem (McCabe et al. (1988)). Following established methods of biolistic bombardment, one can expect recovery of transformed soyben plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

[00341] 11.5 Transformation Method 4. Whiskers mediated transformation.

[00342] Whisker preparation and whisker transformation can anticipated according to methods described previously by Terakawa et al. (2005)). Following established methods of biolistic bombardment, one can expect recovery of transformed soyben plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

[00343] Maverick seeds were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 min. and then rinsed three times in sterile distilled water. The seeds were soaked in distilled water for 18–20 h. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were positioned in the bombardment medium [BM: MS (Murashige and Skoog 1962) basal salts medium, 3% sucrose and 0.8% phytagel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium.

[00344] 11.6 Transformation Method 5. Particle bombardment-mediated transformation for embryogenic callus tissue can be optimized for according to previous methods (Khalafalla et al., 2005; El-Shemy et al., 2004, 2006). Regenrated plants can also be assessed according to Example 11.2.

Example 12– *AAD-12 (v1)* in Cotton

[00345] 12.1 – Cotton transformation protocol.

[00346] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (Table 26) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00347] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 26) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (Table 26) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 26). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00348] *Agrobacterium* preparation. Inoculate 35ml of Y media (Table 26) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (Table 26) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per ml with a total volume of 40 ml.

[00349] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison, MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.01 and 0.05mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for to the AAD-12 enzyme, however dichlorprop is more active on cotton than 2,4-D. In a separate comparison, segments which were plated on MG media containing no growth regulator compared to standard MG media, showed reduced callusing, but there still is callus growth. Callus is then transferred to CG-media (Table 26), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 26) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed,

and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color. Cotton can take time to regenerate and form embryos, one of the ways to speed up this process is to stress the tissue. Dessication is a common way to accomplish this, via changes in the microenvironment of the tissue and plate, by using less culture media and/or adopting various modes of plate enclosure (taping versus parafilm).

[00350] Larger, well-developed embryos are isolated and transferred to DK media (Table 26) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

Table 26. Media for Cotton Transformation

Ingredients in 1 liter	**G**	**M liquid**	**M**	**MG**	**CG**	**D**	**DK**	**Y**
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
Agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA				2 ml	0.4 ml			
Carbenicillin (250 mg/ml)				0.5 ml	0.3 ml			10 grams
GLA (10mg/ml)								10 grams
Peptone								5 grams
Yeast Extract								
NaCl								

[00351] 12.2 – Cell transformation.

[00352] Several experiments were initiated in which cotyledon segments were treated with *Agrobacterium* containing pDAB724. Over 2000 of the resulting segments were treated using various auxin options for the proliferation of pDAB724 cotton callus, either: 0.1 or 0.5mg/L R-dichlorprop, standard 2,4-D concentration and no auxin treatment. The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage; then callus lines that are embryogenic will be sent for Western analysis, essentially as described in section 11.2.3. Embryogenic cotton callus was stressed using dessication techniques to improve the quality and quantity of the tissue recovered.

[00353] Almost 200 callus events have been screened for intact PTU and expression using Western analysis for the *AAD-12 (v1)* gene. Below is a subset of the data for some of the cotton callus that has been tested.

Construct	Line Number	*AAD-12* PTU	*AAD-12* Invader	AAD-12 ng/ml
pDAB724	1	+	+	79.89
pDAB724	2	+	+	17.34
pDAB724	3	+	+	544.80
pDAB724	4	+	+	32.63
pDAB724	5	+	+	82.77
pDAB724	83	+	+	795.50
pDAB724	84	+	+	613.35
pDAB724	85	+	+	1077.75
pDAB724	86	+	+	437.74
pDAB724	87	+	+	286.51
pDAB724	88	+	+	517.59
pDAB724	89	+	+	1250.70

[00354] 12.3 – Plant regeneration.

[00355] *AAD-12 (v1)* cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the *AAD-12 (v1)* gene provides resistance to 2,4-D in cotton, both the *AAD-12 (v1)* cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187 L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T_1 seed or outcrossed with an elite cotton line to produce F_1 seed. The subsequent seed produced will be planted and evaluated for herbicide

resistance as previously described. AAD-12 (v1) events can be combined with other desired HT or IR trants as described in experimens 18, 19, 22, and 23.

Example 13 – *Agrobacterium* Transformation of Other Crops

[00356] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g.,* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.*, 1997.

[00357] The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *AAD-12 (v1)*, for example, into these and other plants, including but not limited to Maize (*Zea mays*), Wheat (*Triticum* spp.), Rice (*Oryza* spp. and *Zizania* spp.), Barley (*Hordeum* spp.), Cotton (*Abroma augusta* and *Gossypium* spp.), Soybean (*Glycine max*), Sugar and table beets (*Beta spp.*), Sugar cane (*Arenga pinnata*), Tomato (*Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato (*Solanum tubersoum)*, Sweet potato (*Ipomoea betatas)*, Rye (*Secale* spp.), Peppers (*Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Lactuca sativa*, *perennis*, and *pulchella*), Cabbage (*Brassica spp*), Celery (*Apium graveolens*), Eggplant (*Solanum melongena*), Peanut (*Arachis* hypogea), Sorghum (all *Sorghum* species), Alfalfa (*Medicago sativua*), Carrot (*Daucus carota*), Beans (*Phaseolus* spp. and other genera), Oats (*Avena sativa* and *strigosa*), Peas (*Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Helianthus annuus*), Squash (*Cucurbita* spp.), Cucumber (*Cucumis sativa*), Tobacco (*Nicotiana* spp.), *Arabidopsis* (*Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, *Poa, Cynadon,* and other genera), Clover (*Tifolium*), Vetch (*Vicia*). Such plants, with *AAD-12 (v1)* genes, for example, are included in the subject invention.

[00358] *AAD-12 (v1)* has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluroxypyr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited

to: Alder (*Alnus spp.*), ash (*Fraxinus spp.*), aspen and poplar species (*Populus spp.*), beech (*Fagus spp.*), birch (*Betula spp.*), cherry (*Prunus spp.*), eucalyptus (*Eucalyptus spp.*), hickory (*Carya spp.*), maple (*Acer spp.*), oak (*Quercus spp*), and pine (*Pinus spp*). Use of auxin resistance for the selective weed control in ornamental and fruit-bearing species is also within the scope of this invention. Examples could include, but not be limited to, rose *(Rosa spp.),* burning bush (*Euonymus spp.*), petunia (*Petunia spp)*, begonia (*Begonia spp.*), rhododendron (*Rhododendron spp),* crabapple or apple (*Malus spp.*), pear (*Pyrus spp.*), peach (*Prunus spp*), and marigolds (*Tagetes spp.*).

Example 14 – Further Evidence of Surprising Results: *AAD-12 vs. AAD-2*

[00359] 14.1 – *AAD-2 (v1)* initial cloning.

[00360] Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00361] The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC

TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

[00362] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H_2O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00363] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO:6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:7) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[00364] 14.2 – Completion of *AAD-2 (v1)* binary vector.

[00365] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. *See* PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:14) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:15) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing

reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI – NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

[00366] 14.3 – Evaluation of transformed *Arabidopsis*.

[00367] Freshly harvested T_1 seed transformed with a plant optimized *AAD-12 (v1)* or native *AAD-2 (v1)* gene were planted and selected for resistance to glufosinate as previously described Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

[00368] *AAD-12 (v1)* and *AAD -2 (v1)* did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Surprisingly, *AAD-2 (v1)*and *AAD-2 (v2)* transformants were far less resistant to 2,4-D than the *AAD-12 (v1)* gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (*see* PCT/US2005/014737). Conversely, *AAD-12 (v1)* had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D (Table 11). Tolerance improved slightly for plant-optimized *AAD-2 (v2)* versus the native gene; however, comparison of both *AAD-12* and *AAD-2* plant optimized genes indicates a significant advantage for *AAD-12 (v1) in planta.*

[00369] These results are unexpected given that the *in vitro* comparison of *AAD-2 (v1)* (*see* PCT/US2005/014737) and *AAD-12 (v2)* indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual T_1 plants to varying levels; however, little protection

from 2,4-D injury is afforded by this expressed protein. No substantial difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes (*see* PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of *AAD-12 (v1) in planta*, and resulting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides, unexpected.

Example 15 – Preplant Burndown Applications

[00370] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

[00371] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (*see* federal labels, most are available through

CPR, 2005 or online at cdms.net/manuf/manuf.asp). With *AAD-12 (v1)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2005) and uses described in Agriliance Crop Protection Guide (2005), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. Likewise, the unique attributes of AAD-12 allowing degradation of triclopyr and fluroxypyr increase utility by allowing substitution or tank mixesof 70-1120 or 35-560 g ae/ha of triclopyr and fluroxypyr, respectively, to increase spectram and/or increase the abilty to control perennial or viney weed species.

Example 16 – In-Crop Use of Phenoxy Auxins Herbicides in Soybeans, Cotton, and Other Dicot Crops Transformed Only with *AAD-12 (v1)*

[00372] *AAD-12 (v1)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D and MCPA) and pyridyloxy auxins (triclopyr and fluroxypyr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00373] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluroxypyr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

[00374] A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetmide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

[00375] Further benefits could include tolerance to 2,4-D, triclopyr or fluroxypyr required before planting following aryloxyacetic acid auxin herbicide application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D, triclopyr or fluroxypyr. Dicamba (and many other herbicides) can still be used for the subsequent control of *AAD-12 (v1)*-transformed dicot crop volunteers.

[00376] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by *AAD-12 (v1)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 17 - In-Crop Use of Phenoxy Auxin and Pyridyloxy Auxin Herbicides in *AAD-12 (v1)* Only Transformed Corn, Rice, and Other Monocot Species

[00377] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-12 (v1)* would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D,.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. *AAD-12 (v1)*-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00378] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, or fluroxypyr. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-12 (v1)* HTC combination strategy, whether one rotates crops species or not.

[00379] A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetmide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

[00380] The increased tolerance of corn, rice, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage

restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 18 – *AAD-12 (v1)* Stacked With Glyphosate Tolerance Trait in Any Crop

[00381] The vast majority of cotton, canola, corn, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-12 (v1)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed,

or as a premix with glyphosate to provide effective control. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-12 (v1)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Tricopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimin. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D, triclopyr, or fluroxypyr.

[00382] One skilled in the art of weed control will recognize that use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 19 - *AAD-12 (v1)* Stacked with Glufosinate Tolerance Trait in Any Crop

[00383] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and

broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2005).

[00384] By stacking *AAD-12 (v1)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis Apocynum cannabinum, and Conyza candensis*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species. Triclopyr and fluroxypyr would be acceptable components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) A multiple combination of glufosinate (200-500 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00385] One skilled in the art of weed control will recognize that use of one or more commercial aryloxyacetic auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - *AAD-12 (v1)* Stacked with *AHAS* Trait in Any Crop

[00386] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2005). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2005). By stacking *AAD-12 (v1)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monoct crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and these auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2005) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp.* can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. See rates above for triclopyr or fluroxypyr.

b) A mulitple combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00387] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by *AAD-12 (v1)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-12 (v1)* in Rice

[00388] 21.1 – Media description.

[00389] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/L Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

[00390] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/L glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/L sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/L hygromycin B for 3–4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/L 6-benzylaminopurine (BAP), 1 mg/L α-naphthaleneacetic acid (NAA), 5 mg/L abscisic acid (ABA) and 50 mg/L hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/L BAP, 0.5 mg/L NAA, and 50 mg/L hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/L hygromycin B (1/2MSH50).

[00391] 21.2 – Tissue culture development.

[00392] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that

the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week. prior to storage.

[00393] 21.3 – Microprojectile bombardment.

[00394] All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(*Hpt*-containing vector) to pDAB4101 (*AAD-12 (v1)* + *AHAS*), 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[00395] 21.4 – Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T_0 Rice

[00396] Rice plantlets at the 3-5 leaf stage were sprayed with a lethal dose of 0.16% (v/v) solution of Pursuit (to confirm the presence of the *AHAS* gene) containing 1% Sunit II (v/v) and 1.25% UAN (v/v) using a track sprayer calibrated to 187 L/ha. Rating for sensitivity or resistance was performed at 36 days after treatment (DAT). Ten of the 33 events sent to the greenhouse were robustly tolerant to the Pursuit; others suffered varying levels of herbicide injury. Plants were sampled (according to section 21.7 below) and molecular characterization

was performed as previously described in Example 8 that identified seven of these 10 events as containing both the *AAD-12 (v1)* PTU and the entire *AHAS* coding region.

[00397] 21.5 – Heritability of AAD-12 (v1) in T_1 rice

[00398] A 100-plant progeny test was conducted on five T_1 lines of *AAD-12 (v1)* lines that contained both the *AAD-12 (v1)* PTU and *AHAS* coding region. The seeds were planted with respect to the procedure above and sprayed with 140 g ae/ha imazethapyr using a track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Two out of the five lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 coseregated with the *AHAS* selectable marker as determined by 2,4-D tolerance testing below.

[00399] 21.6 – Verification of high 2,4-D tolerance in T_1 Rice.

[00400] The following T_1 *AAD-12 (v1)* single segregating locus lines were planted into 3-inch pots containing Metro Mix media: pDAB4101(20)003 and pDAB4101(27)002. At 2-3 leaf stage were sprayed with 140 g ae/ha imazethapyr. Nulls were eliminated and individuals were sprayed at V3-V4 stage in the track sprayer set to 187 L/ha at 1120, 2240 or 4480 g ae/ha 2,4-D DMA (2X, 4X, and 8X typical commercial use rates, respectively). Plants were graded at 7 and 14 DAT and compared to untransformed commercial rice cultivar, 'Lamont,' as negative control plants.

[00401] Injury data (Table 27) shows that the *AAD-12 (v1)*-transformed lines are more tolerant to high rates of 2,4-D DMA than the untransformed controls. The line pDAB4101(20)003 was more tolerant to high levels of 2,4-D than the line pDAB4101(27)002. The data also demonstrates that tolerance of 2,4-D is stable for at least two generations.

Table 27. T_1 *AAD-12 (v1)* and untransformed control respone to varying levels of 2,4-D DMA.

Herbicide	Lemont Untransformed Control	pDAB4101(20)003	pDAB4101(27)002
	Average % Injury 14 DAT		
1120 g ae/ha 2,4-D DMA	20	10	10
2240 g ae/ha 2,4-D DMA	35	15	30
4480 g ae/ha 2,4-D DMA	50	23	40

[00402] 21.7 – Tissue harvesting, DNA isolation and quantification.

[00403] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

[00404] 21.8 – AAD-12 (v1) expression.

[00405] Sample preparation and analysis conditions were as described previously. All 33 T_0 transgenic rice lines and 1 non-transgenic control were analyzed for AAD-12 expression using ELISA blot. AAD-12 was detected in the clones of 20 lines, but not in line Taipai 309 control plant. Twelve of the 20 lines that had some of the clones tolerant to imazethapyr were expressing AAD-12 protein, were AAD-12 PCR PTU positive, and AHAS coding region positive. Expression levels ranged from 2.3 to 1092.4 ppm of total soluble protein.

[00406] 21.9– Field tolerance of pDAB4101 rice plants to 2,4-D and triclopyr herbicides.

[00407] A field level tolerance trial was conducted with *AAD-12 (v1)* event pDAB4101[20] and one wild-type rice (Clearfield 131) at Wayside, Mississippi (a non-transgenic imidazolinone-resistant variety). The experimental design was a randomized complete block design with a single replication. Herbicide treatments were 2X rates of 2,4-D (dimethylamine salt) at 2240 g ae/ha and triclopyr at 560 g ae/ha plus an untreated control. Within each herbicide treatment, two rows of T_1 generation pDAB4101[20] and two rows of Clearfield rice were planted using a small plot drill with 8-inch row spacing. The pDAB4101[20] rice contained the *AHAS* gene as a selectable marker for the *AAD-12(v1)* gene. Imazethapyr was applied at the one leaf stage as selection agent to remove any *AAD-12 (v1)* null plants from the plots. Herbicide treatments were applied when the rice reached the 2 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual ratings of injury were taken at 7, 14 and 21 days after application.

[00408] *AAD-12 (v1)* event response to 2,4-D and triclopyr are shown in Table 28. The non-transformed rice line (Clearfield) was severely injured (30% at 7DAT and 35% at 15DAT) by 2,4-D at 2240 g ae/ha which is considered the 4X commercial use rate. The *AAD-12 (v1)*

event demonstrated excellent tolerance to 2,4-D with no injury observed at 7 or 15DAT. The non-transformed rice was significantly injured (15% at 7DAT and 25% at 15DAT) by the 2X rate of triclopyr (560 g ae/ha). The *AAD-12 (v1)* event demonstrated excellence tolerance to the 2X rates of triclopyr with no injury observed at either 7 or 15DAT.

[00409] These results indicate that the *AAD-12 (v1)* transformed rice displayed a high level of resistance to 2,4-D and triclopyr at rates that caused severe visual injury to the Clearfield rice. It also demonstrates the ability to stack multiple herbicide tolerance genes with AAD-12 I multiple species to provide resistance to a wider spectrum of effective chemistries

Table 28. AAD-12 T_1 generation rice plants response to 2,4-D and triclopyr under field conditions.

Herbicide Treatment		% Visual Injury			
		7DAT		15DAT	
Active Ingredient	Rate	AAD-12 event pDAB4101[20]	Wild-type Clearfield	AAD-12 event pDAB4101[20]	Wild-type Clearfield
2,4-D	2240 GM AE/HA	0	15	0	35
Triclopyr	840 GM AE/HA	0	30	0	25
Untreated		0	0	0	0

Example 22 – *AAD-12 (v1)* in Canola

[00410] 22.1 – Canola transformation.

[00411] The *AAD-12 (v1)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained *AAD-12 (v1)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi10 promoter (*see* section 2.4).

[00412] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00413] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/L kinetin and 1 mg/L 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00414] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/L Carbenicillin and 300 mg/L Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/L Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00415] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 1 mg/L Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 3 mg/L Herbiace, Carbenicillin and Timentin) for another 2-3 weeks.

[00416] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/L MES, 5 or 10 mg/L Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/L Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00417] 22.2 – Molecular analysis: canola materials and methods

[00418] 22.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green

(Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ul.

[00419] 22.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-12 (v1)* were (SEQ ID NO:10) (forward) and (SEQ ID NO:11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-12 (v1)* events tested positive. Three negative control samples tested negative.

[00420] 22.2.3 – ELISA.

[00421] Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 29.

Table 29. Expression of *AAD-12 (v1)* in Canola plants

Sample #	[TSP] (µg/ml)	[AAD-12] (ng/ml)	Expression (ppm TSP) (ELISA)	Western
31	5614.96	1692.12	301.36	++++
33	4988.26	2121.52	425.30	++++
38	5372.25	3879.09	722.06	++++
39	2812.77	41.36	14.71	+
40	3691.48	468.74	126.98	+++
Control 1	2736.24	0.00	0.00	-
Control 2	2176.06	0.00	0.00	-
Control 3	3403.26	0.00	0.00	-

[00422] 22.4 – Postemergence herbicide tolerance in *AAD-12(v1)* transformed T_0 Canola.

[00423] Forty-five T_0 events from the transformed with the construct pDAB3759, were sent to the greenhouse over a period of time and were allowed to acclimate in the greenhouse. The plants were grown until 2-4 new, normal looking leaves had emerged (i.e., plants had

transitioned from tissue culture to greenhouse growing conditions). Plants were then treated with a lethal dose of the commercial formulations of 2,4-D Amine 4 at a rate of 560 g ae/ha. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. A lethal dose is defined as the rate that causes >95% injury to the untransformed controls.

[00424] Twenty-four of the events were tolerant to the 2,4-D DMA herbicide application. Some events did incur minor injury but recovered by 14 DAT. Events were progressed to the T_1 (and T_2 generation) by selfpollination under controlled, bagged, conditions.

[00425] 22.5 – *AAD-12 (v1)* heritability in Canola.

[00426] A 100 plant progeny test was also conducted on 11 T_1 lines of *AAD-12 (v1)*. The seeds were sown and transplanted to 3-inch pots filled with Metro Mix media. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Seven out of the 11 lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi-square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species and can be stacked with one or more additional herbicide resistance genes.

[00427] 22.6 – Verification of high 2,4-D tolerance in T_1 Canola

[00428] For T_1 *AAD -12 (v1)*, 5-6 mg of seed were stratified, sown, and a fine layer of Sunshine Mix # 5 media was added as a top layer of soil. Emerging plants were selected with 560 g ae/ha 2,4-D at 7 and 13 days after planting.

[00429] Surviving plants were transplanted into 3-inch pots containing Metro Mix media. Surviving plants from T1 progenies, that were selected with 560 g ae/ha 2,4-D, were also transplanted into 3-inch pots filled with Metro Mix soil. At 2-4 leaf stage plants were sprayed with either 280, 560, 1120, or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to untransformed control plants. A sampling of T_1 event injury data 14DAT may be seen in **Table 30**. Data suggests that multiple events are robustly resistant to 2240 g ae/ha 2,4-D, while other events demonstrated less robust tolerance up to 1120 g ae/ha 2,4-D. Surviving plants were transplanted to 5 1/4" pots containing Metro Mix media and placed in the same growth conditions as before and self-pollinated to produce only homozygous seed.

Table 30. T_1 *AAD-12 (v1)* and untransformed control response to varying rates postemergence 2,4-D DMA applications.

Herbicide	Untransformed Control	pDAB3759(33)013.001	pDAB3759(18)009.001	pDAB3759(18)022.001	pDAB3759(18)030.001	pDAB3759(18)023.001
	Average % Injury 14DAT					
280 g ae/ha 2,4-D DMA	85	0	0	0	0	0
560 g ae/ha 2,4-D DMA	85	0	0	0	0	0
1120 g ae/ha 2,4-D DMA	90	0	0	13	5	3
2240 g ae/ha 2,4-D DMA	95	1	5	83	31	6

[00430] 22.7-Field tolerance of pDAB3759 canola plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

[00431] Field level tolerance trial was conducted on two *AAD-12 (v1)* events 3759(20)018.001 and 3759(18)030.001 and a wild-type canola (Nex710) in Fowler, Indiana. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Within each herbicide treatment, single 20 ft row/event for event 3759(18)030.0011, 3759(18)018.001 and wild-type line (Nex710) were planted with a 4 row drill on 8 inch row spacing. Herbicide treatments were applied when canola reached the 4-6 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after application.

[00432] Canola response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 31. The wild-type canola (Nex710) was severely injured (72% at 14DAT) by 2,4-D at 2240 g ae/ha which is considered the 4X rate. The *AAD-12 (v1)* events all demonstrated excellent tolerance to 2,4-D at 14DAT with an average injury of 2, 3 and 2% observed at the 1, 2 and 4X rates, respectively. The wild-type canola was severely injured (25% at 14DAT) by the 2X rate of triclopyr (840 g ae/ha). *AAD-12 (v1)* events demonstrated tolerance at 2X rates of triclopyr with an average of 6% injury at 14DAT across the two events. Fluroxypyr at 280 g ae/ha

caused severe injury (37%) to the non-transformed line at 14DAA. *AAD-12 (v1)* events demonstrated increased tolerance with an average of 8% injury at 5DAT.

[00433] These results indicate that *AAD-12 (v1)* transformed events displayed a high level of resistance to 2,4-D, triclopyr and fluroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed canola. AAD-12 has been shown to have relative efficacy of 2,4-D > triclopyr > fluroxypyr.

Table 31. AAD-12 (pDAB3759) canola plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

Herbicide Treatment		% Visual Injury at 14 DAT		
Active Ingredient	Rate	AAD-12 event 3759(20)018.001	AAD-12 event 3759(18)030.001	Wild Type (Nex710)
2,4-D	280 GM AE/HA	0 a	0 b	0 e
2,4-D	560 GM AE/HA	0 a	0 b	15 d
2,4-D	1120 GM AE/HA	2 a	2 ab	33 bc
2,4-D	2240 GM AE/HA	3 a	3 ab	48 a
Triclopyr	840 GM AE/HA	6 a	6 ab	25 cd
Fluroxypyr	280 GM AE/HA	7 a	8 a	37 ab

Means with a column with different letters are significantly different as defined by LSD (p=0.05).

Example 23--*AAD-12 (v1)* Stacked With Insect Resistance (IR) or Other Input Traits in Any Crop

[00434] Insect resistance in crops supplied by a transgenic trait is prevelant in corn and cotton production in North America and across the globe. Commercial products having combined IR and HT traits have been developed by multiple seed companies. These include Bt IR traits (e.g. Bt toxins listed at the website lifesci.sussex.ac.uk, 2006) and any or all of the HTC traits mentioned above. The value this offering brings is the ability to control multiple pest problems through genetic means in a single offering. The convenience of this offering will be restricted if weed control and insect control are accomplished independent of each other. *AAD-12 (v1)* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input traits (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) (isb.vt.edu/cfdocs/fieldtests1.cfm, 2005) either through conventional breeding or jointly as a novel transformation event. Benefits include the convenience and flexibility described in Examples 15-20 above, together with the ability to manage insect pests and/or other

agronomic stresses in addition to the improved weed control offered by *AAD-12* and associated herbicide tolerance. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues.

[00435] Combined traits of IR and HT have application in most agronomic and horticultural/ornamental crops and forestry. The combination of *AAD-12* and its commensurate herbicide tolerance and insect resistance afforded by any of the number of Bt or non-Bt IR genes are can be applied to the crop species listed (but not limited to) in Example 13. One skilled in the art of weed control will recognize that use of any of various commercial herbicides described in Examples 18-20, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by *AAD-12 (v1)* transformation and stacking with the corresponding HT trait or IR trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2005). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 24—*AAD-12 (v1)* as an *in vitro* Dicot Selectable Marker

[00436] Genetic engineering of plant cell, tissue, organ, and plant or organelle such as plastid starts with the process of inserting genes of interest into plant cells using a suitable delivery method. However, when a gene is delivered to plant cells, only an extremely small percentage of cells integrate the heterogeneous gene into their genome. In order to select those few cells that have incorporated the gene of interest, researchers link a selectable or screenable "marker gene" to the gene of interest (GOI) in the vector. Cells that contain these markers are identified from the whole population of cells/ tissue to which the DNA plasmid vector was delivered. By selecting those cells that express the marker gene, researchers are able to identify those few cells that may have incorporated the GOI into their genome.

[00437] There are a variety of selectable markers available to enable this selection process to obtain transgenic cells, callus, embryos, shoots and plantlets. The preferred selectable markers by the Ag-industry are herbicide markers that allow the ease of spraying compounds

in the field to select the right transgenic progenies during the process of event sorting in the field situation. *AAD-12 (v1)* has been shown to efficiently serve as a selectable marker for whole plants transformed with the gene in the greenhouse and growth chamber (Example 7) with 2,4-D as the selection agent. Field selection is possible as well using 2,4-D in combination with the *AAD-12 (v1)* gene (Example 11, 22), but use *in* vitro for cell-level selection is complicated by the fact 2,4-D is used almost ubiquitously as a plant growth regulator in the plant tissue culture systems. Degradation of this important hormone by AAD-12 (v1) can impact he ability to use this gene as an in vitro selectable marker. Success of developing 2,4-D as a marker gene depends on identifying the right alternate plant growth regulator that can mimic the effect of 2,4-D in the respective culture system and at the same time possess the ability to be stable and not be degraded by the AAD-12 enzyme when expressed in the transgenic cells. R-dichlorprop is a close analog to 2,4-D that is not a substrate for *AAD-12 (v1)* and is used a non-metabolizable auxin substitute in tobacco cell cultures allowing 2,4-D to be used at high rates as a selection agent. This fact was used in exemplifying *AAD-12 (v1)* could be used as a selectable marker *in vitro*.

[00438] 24.1—Cell culture--Alternative auxins.

[00439] AAD-12 (v1) degrades 2,4-D, but not R-2,4-dichlorophenoxypropionic acid (R-dichlorprop), which has at the same time the structural requirement of an auxinic growth regulator. Other non-metabolizable plant auxin mimics that may be used in cell culture include NAA (naphthalene acetic acid), IAA (indole acetic acid), dicamba, picloram, and R-mecoprop. It was investigated if it was possible to substitute R-dichlorprop and successfully maintain two different tobacco cell cultures PHL (Petite Havanna) and BY2 suspensions. Conversely, for cotton explants R-dichlorprop, dicamba, and picloram were tested as alternative auxins and the embryogenic callus induction response in comparison to the standard growth regulator, 2,4-D was evaluated. Petite Havana tobacco (PHL) and Coker cotton cotyledons were used in their experiments.

[00440] 24.1.1—Tobacco cell suspension—2,4-D as selection agent.

[00441] A dose response study was conducted with both the R-dichlorprop habituated PHL cells and the R-dichlorprop habituated BY2 cells where R-dichlorprop was substituted directly for 2,4-D in culture media. Though the focus was on PHL, a dose response was also done with BY2 in case of possible future studies, as well as to help predict the dose response for PHL. For the dichlorprop habituated PHL dose response, the levels of 2,4-D used (on

LSBY2C medium with R-dichlorprop) were 0 (the control), 1, 2, 3, 5, 8, 10, 12, 15, 18, 20, 40, 60, 80,10,110,120 mg/L 2,4-D. There were four replications per concentration. For the R-dichlorprop habituated BY2 dose response, the levels of 2,4-D used (on LSBY2C medium) were 0 (the control), 1, 2, 3, 5, 8, 10, 20, 30,40, mg/L 2,4-D.

[00442] The dose response was carried out showed that all the concentration of 2,4-D tested were lethal above 10 mg/ L concentrations. However, there was growth in all the concentrations up to 10-mg/L 2,4-D where a slight growth of PHL suspension was observed. The growth of the suspension colonies from 1- 8 mg/L 2,4-D concentrations was comparable to the growth in control treatments. The observation made in BY2 suspension cells were similar except the concentration at 10 mg/L was found to be lethal and the sub lethal concentration was 8 mg/L concentration.

[00443] 24.1.2—Tobacco cell transformation with *AAD-12 (v1)* and 2,4-D selection.

[00444] For tobacco transformation experiment, there were 11 treatments altogether: a control set plated on LS-BY2C + dichlorprop medium , and 10 sets of LSBY2C + dichlorprop + 2,4-D at varying concentration levels (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L). There were four replications per treatment. The plasmid DNA vector used was pDAB724, and the vector used for transformation was EHA101S strain of Agrobacterium tumefaciens. Four ml of PHL suspension at 0.6 $0D^{660}$ were mixed with 100 ul of Agrobacterium (either EHA101 or LBA4404 strains) suspension at 1.0 $0D^{660}$ in a sterile Petri plate and were mixed thoroughly and co-cultivated together in a non-shake condition at a dark growth chamber for 3 days at 25°C . After the co-cultivation period 1.5 ml of the Agro-tobacco suspension mixture was plated to the 11 set of plates above. The experiment was repeated with 13 treatments: a control of LS-BY2C + dichlorprop media (no 2,4-D), and LS-BY2C + dichlorprop + 2,4-D (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L); LSBY2C + 1mg/L 2,4-D + B10 (Bialophos); LSBY2C + 10 2,4-D + B10 + R-dichlorprop. Again, there were four replicates per treatment, as well as a positive and negative control. All media contain 500 mg/L Carbenicillin (C) to control to contain Agrobacterium growth in the selection media.

[00445] The plasmid used in these experiments is pDAB724 and it has *PAT* selectable marker as well. So, control transformation experiments were initiated using R-dichlorprop habituated PHL in the presence of 10 mg/L bialophos following the standard protocol described above. The treatments were done side by side with 4 replicates to see if the bialophos selection in these suspension is normal.

[00446] There was little growth observed in all selection concentrations of 2,4D tested above 10 mg/L; however several fast growing colonies were found in 2, 5, an 8 mg/L 2,4-D concentration and representative sample was transferred to fresh selection at 10 mg/L selection to bulk the callus. Also, several putative colonies were selected in from 12, 15,18 and 20 mg/L 2,4-D, but when compared to 10 mg/L there were only few colonies in these selection plate. Control treatment conducted with bialophos selection showed normal colony development. It appears that 10 mg/L 2,4-D is the sub-lethal and above this concentration 2,4-D appears to be lethal to the non-transformed cells. All the identified colonies were transferred to fresh medium with 10 mg/L selection and were probed for the presence of transgene by PCR as described in Example 10. The colonies selected and bulked had the transgenes as determined by PCR and expression of the genes as established by the Western analyses (as described in example 10). Several colonies were identified as actively growing and transferred to fresh selection medium with 10 mg/L 2,4-D to bulk the callus.

[00447] The bulked calluses were then transferred to higher level of 2,4-D to test the tolerance level *in vitro*. The levels of 2,4-D used were 20, 40, 60, 80, 100, and 120 mg/L 2,4-D. However the callus did not grow beyond 20 mg/L 2, 4-D concentrations indicating a threshold concentration higher than 20 mg/L may exist.

[00448] 24.2.1— Cotton explants—auxin alternatives

[00449] A dose response study was initiated to test multiple auxin alternatives as a substitute for the use of 2,4-D as a growth regulator in cotton. The alternative auxin tested were 2,4-dichlorprop, dicamba, and picloram. These compounds were tested at 0.2, 2.0, and 20.0 uM concentrations respectively. 2,4-D was used as the control treatment at 0.02 uM concentration. The medium used is the base medium for cotton callus induction (Example 12). Beyond the initial phase of culture, auxin is removed from the medium to prod the tissue toward the regeneration process.

[00450] R-dichlorprop was not effective in callus induction of cotyledonary segments and appears toxic to cotton cells at the lowest concentration tested (0.02 uM). Dicamba effectively induces callus growth at all concentrations tested (0.02-20 uM) and has no apparent toxic effects in this concentration range. Callus induction with picloram increased up to a maximum when explants were treated with 0.2 uM to 20 uM. Quality of the callus was consistent with the standard 2,4-D treatment at the 2 uM picloram concentration. At the

highest concentration (20 uM) 2,4-D was also inhibitory to cotton callus generation and growth.

[00451] Cotton has shown initial ability to respond effectively to alternative auxins (to 2,4-D) in culture. At high enough concentrations, 2,4-D is toxic to cotton cotyldeonary explants. R-dichlorprop is surprisingly significantly more toxic to cotton than 2,4-D or other auxins. 2,4-D may be used as a selection agent and in combination with *AAD-12 (v1)* as the selectable marker gene. Other non-metabolizable auxin surrogates (e.g., dicamba, picloram, R-mecoprop, NAA, or IAA) would allow the use of AAD-12 as a selectable marker in dicots with 2,4-D as the selection agent.

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Claims

We claim:

1. A transgenic plant cell comprising a polynucleotide that encodes an AAD-12 protein selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, a variant of SEQ ID NO:2, and a variant of SEQ ID NO:4, said variant having aryloxyalkanoate dioxygenase activity and at least 95% sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

2. The cell of claim 1 wherein expression of said polynucleotide imparts to said cell an ability to convert a phenoxyacetate auxin herbicide to a non-herbicidal molecule.

3. The cell of claim 1 wherein expression of said polynucleotide imparts to said cell an ability to convert a pyridyloxy auxin herbicide to a non-herbicidal molecule.

4. A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said plant tolerant to said herbicide.

5. A method of selecting for at least one transgenic plant cell of claim 1 wherein said method comprises applying an aryloxyalkanoate herbicide on and/or about said transgenic plant cells and to lacking plant cell that lack said polynucleotide, and allowing said transgenic plant cell and said lacking plant cells to grow.

6. The method of claim 5 wherein said transgenic plant cell is a plant cell of a transgenic crop plant growing in a field, and said lacking cells are cells of a weed growing in said field.

7. The method of claim 5 wherein said transgenic plant cell is a plant cell transformed with said polynucleotide, and said lacking cells are non-transformants, and all of said cells are grown on tissue culture medium.

8. A method of controlling weeds, said method comprising applying a 2,4-D herbicide to a crop plant and to a weed, said crop plant comprising a plurality of plant cells according to claim 1.

9. The method of claim 8, wherein said crop plant is a soybean plant.

10. The method of claim 8, wherein said weed is resistant to glyphosate.

11. A method of protecting a crop plant from damage by an aryloxyalkanoate dioxygenase herbicide, said method comprising growing a plant comprising an AAD-12 gene, and applying said herbicide to said plant.

12. The method of claim 11 wherein said herbicide is 2,4-D.

13. The method of claim 8 wherein said plant further comprises a glyphosate resistance gene, and said method further comprises applying glyphosate to said plant and to said weed.

14. The method of claim 13, said plant further comprising a third herbicide resistance gene, and said method further comprising applying a third herbicide to said plant and to said weed.

15. A transgenic plant comprising a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide.

16. The transgenic plant of claim 15, said polynucleotide also conferring resistance to a phenoxy auxin herbicide.

17. The plant of claim 16, said phenoxy auxin herbicide selected from the group consisting of 2,4-D and MCPA.

18. The plant of claim 15, said plant further comprising at least one additional herbicide resistance gene.

19. A plant comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

20. A polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said polynucleotide comprises a codon composition that is optimized for expression in a plant.

21. A plant cell comprising a heterologous polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

22. A method of selecting for a transformed plant cell of claim 21 wherein said method comprises subjecting a plurality of plant cells to transformation with said polynucleotide, then growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin herbicide.

23. The method of claim 22 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

24. A method of controlling at least one weed in a field, said method comprising planting in said field seed of at least one transgenic plant, said plant comprising

a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide, and

a second heterologous polynucleotide encodes an enzyme conferring resistance to at least one other herbicide;

said method further comprising applying to at least a portion of said field a first herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide; and applying said at least one other herbicide to said at least a portion of said field.

25. The method of claim 24 wherein said herbicides are applied sequentially or concurrently.

26. The method of claim 24 wherein said first herbicide is a phenoxy auxin herbicide.

27. The method of claim 24 wherein said at least one other herbicide is selected from the group consisting of acetochlor, acifluorfen, alloxydim, amidosulfuron, aminopyralid, atrazine, beflubutamid, bispyribac, butafenacil, cafenstrole, carfentrazone, chlorimuron, chlorotoluron, cinidon-ethyl, clethodim, clodinafop, clomazone, cloproxydim, clopyralid, cloransulam, cyanazine, cyclosulfamuron, cycloxydim, cyhalofop, daimuron, dicamba, diclofop, diclosulam, diflufenican, dimethenamid, diquat, dithiopyr, diuron, ethalfluralin, fenoxaprop, flazasulfuron, florasulam, fluazifop, flucarbazone, flufenacet, flufenican, flufenpyr, flumetsulam, flumiclorac, flumioxazin, fluroxypyr, fluthiacet, fomesafen, foramsulfuron, glufosinate, glyphosate, halosafen, halosulfuron, haloxyfop, imazamethabenz, imazamox, imazapic, imazapyr, imazaquin, imazethapyr, imazosulfuron, iodosulfuron, ioxynil, isoxaben, isoxaflutole, lactofen, linuron, mefenacet, mefluidide, mesosulfuron, mesotrione, metamifop, metazachlor, metosulam, metribuzin, MSMA, napropamide, nicosulfuron, norflurazon, oryzalin, oxadiazon, oxyfluorfen, paraquat, pebulate, pendimethalin, penoxsulam, picloram, picolinafen, pinoxaden, primisulfuron, profoxydim, propanil, pyraflufen, pyrazosulfuron, pyribenzoxim, pyriminobac, pyrithiobac, pyroxasulfone, pyroxsulam, quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxydim, terbacil, thiazopyr, thidiazuron, thiencarbazone, thifensulfuron, thiobencarb, topramezone,

tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflusulfuron, and tritosulfuron.

28. A method of assaying a protein for its ability to cleave a pyridyloxyalkanoate herbicide, said method comprising providing a sample comprising said protein and a pyridyloxy auxin herbicide, and assaying said sample for presence of a chloropyridinol.

29. A method of assaying a protein for aryloxyalkanoate dioxygenase activity wherein said method comprises providing a sample comprising said protein and 2-(2-chloro,4-nitrophenoxy) propionate and/or acetate, and assaying said sample for presence of a 2-chloro,4-nitrophenol.

30. A heritable expression cassette comprising a nucleic acid molecule encoding a protein that enzymatically degrades at least one herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein said nucleic acid molecule hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

31. The expression cassette of claim 30 wherein said promoter is a plant promoter.

32. The expression cassette of claim 30 comprising a promoter selected from a cassava vein mosaic virus promoter, CaMV 35S promoter, Figwort Mosaic Virus promoter, rice actin promoter, phaseolin promoter, *Arabidopsis thaliana* Ubiquitin 10 promoter, maize ubiquitin promoter, *Arabidopsis thaliana* Act2 promoter, *Arabidopsis thaliana* Ubiquitin 11 promoter, and *Arabidopsis thaliana* Ubiquitin 3 promoter.

33. The method of claim 24 wherein said plant is resistant to a herbicide formulation comprising glyphosate.

34. A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said

first herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin.

35. The method of claim 34 wherein said first herbicide is an acid, an inorganic salt, an organic salt, or an ester.

36. The method of claim 34 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

37. The method of claim 36 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate.

38. A method of detecting whether a plant comprises a polynucleotide of claim 20 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

39. The method of claim 38 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

40. The method of 38 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

41. The method of 39 wherein said method comprises using an antibody to detect for the presence of said protein.

42. The plant of claim 15 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

43. The plant of claim 15 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance,

increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

44. A method of controlling at least one weed in a field, said method comprising growing at least one transgenic plant of claim 15 in said field, and applying a pyridyloxy auxin herbicide to at least a portion of said field.

45. A method of controlling at least one weed in a field, said method comprising growing at least one plant of claim 16 in said field, and applying a phenoxy auxin herbicide to at least a portion of said field.

46. The method of claim 44, further comprising applying a phenoxy auxin herbicide to at least a portion of said field.

47. The method of claim 44, said pyridyloxy auxin herbicide selected from the group consisting of fluroxypyr and triclopyr.

48. The method of claim 45, said phenoxy auxin herbicide selected from the group consisting of 2,4-D and MCPA.

49. The method of claim 45 wherein said plant is resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, imazethapyr, chlorsulfuron, dicamba, mesotrione, isoxaflutole, and butafenacil.

50. The method of claim 45 wherein said herbicide is an achiral phenoxy auxin.

51. The method of claim 24 wherein said plant is a monocot.

52. The method of claim 51 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, warm and cool-season turf grass, oats, sorghum, and pasture grasses.

53. The method of claim 24 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

54. The method of claim 53 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

55. A method of conferring herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a polynucleotide encoding an enzyme capable of using a pyridyloxy auxin herbicide and a phenoxy auxin herbicide as a substrate.

56. A method of controlling glyphosate-resistant, naturally glyphosate tolerant, and/or naturally glufosinate tolerant weeds in a field of glyphosate and/or glufosinate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 13, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

57. The method of claim 56 wherein said herbicide is a phenoxy auxin.

58. The method of claim 57 wherein said phenoxy auxin is 2,4-D.

59. A method of controlling weeds that are resistant to an ALS-inhibiting herbicide in a field of crop plants that comprise a polynucleotide of claim 20, said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

60. The cell of claim 1 wherein said plant cell is selected from the group consisting of dicotyledonous cells and monocotyledonous cells.

61. The cell of claim 60 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

62. The cell of claim 60 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

63. The cell of claim 61 wherein said pyridyloxy auxin herbicide is selected from the group consisting of fluroxypyr and triclopyr.

64. The plant of claim 4 wherein expression of said polynucleotide renders said plant resistant to both a phenoxyacetate auxin herbicide and a pyridyloxy auxin herbicide.

65. The method of claim 8 wherein said method comprises applying a second herbicide.

66. The method of claim 22 wherein said 2,4-D herbicide and said second herbicide are applied sequentially.

67. The method of claim 22 wherein said first herbicide and said second herbicide are applied concurrently.

68. The method of claim 8 wherein said plant is resistant to glyphosate.

69. The method of claim 24 wherein said second polynucleotide is a modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase).

70. The method of claim 24 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

71. The method of claim 70 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

72. The method of claim 24 wherein said method further comprises applying a third herbicide.

73. A polynucleotide optimized for expression in a plant wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

74. The polynucleotide of claim 44 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

75. An isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell.

76. The polynucleotide of claim 75 wherein said promoter is a plant promoter.

77. The polynucleotide of claim 76 wherein said promoter is a cassava vein mosaic virus promoter.

78. The method of claim 22 wherein said polynucleotide is used as a selectable marker.

79. A seed comprising a plant cell of claim 1.

80. A plant grown from the seed of claim 79.

81. A regenerable part, progeny, or asexual propagate of the plant of claim 4.

82. The method of claim 8 wherein said method is used to treat or prevent herbicide-resistant weeds.

83. The plant of claim 4 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

84. The plant of claim 4 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

85. A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 20, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

86. The method of claim 85 wherein said herbicide is a phenoxy auxin.

87. The method of claim 25 wherein said herbicides are applied from a tank mix.

88. The method of claim 10 wherein said at least one of said weeds comprises at least one glyphosate resistant volunteer plant of a different species than said transgenic crop.

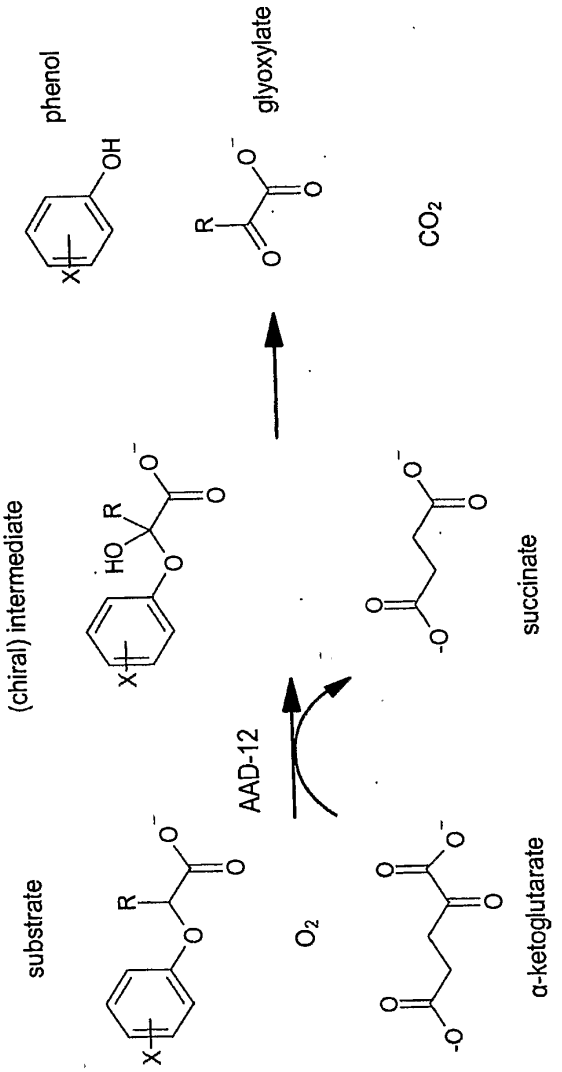

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substrate
AAD-12
O_2
CO_2
α-ketoglutarate

FIG. 1

2/3

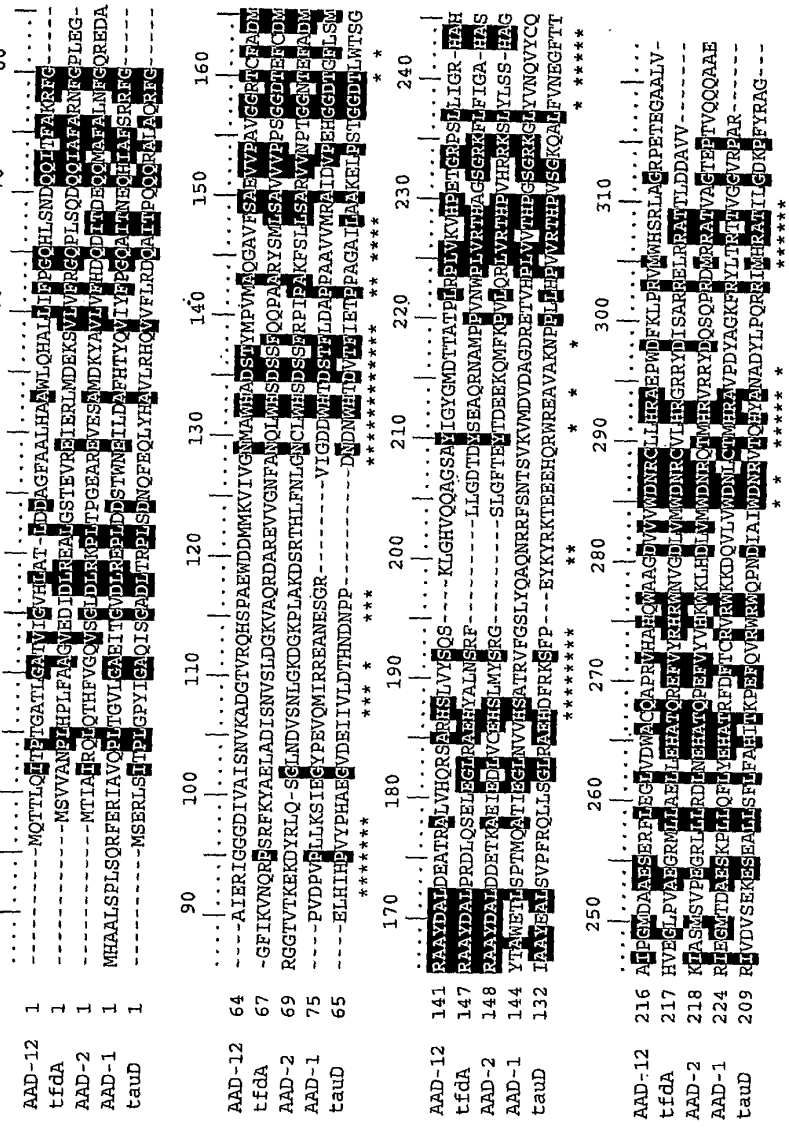


FIG. 2

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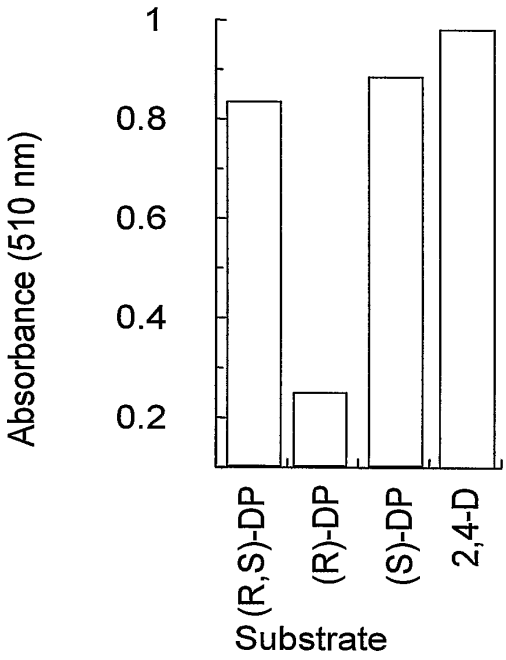


FIG. 3

SEQUENCE LISTING

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Lira, Justin M.
Walsh, Terence A.
Merlo, Donald J.
Jayakumar, Pon Samuel
Lin, Gaofeng

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Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe Gly
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World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1569285

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APPLICATION NUMBER: ***60/731,044***
FILING DATE: ***October 28, 2005***
RELATED PCT APPLICATION NUMBER: ***PCT/US06/42133***

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS ***US60/731,044***

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This is a request for filing PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EQ 005884833 US

112963 U.S. PTO
60773104
102805

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Justin M.	Lira	Indianapolis, IN
Terence A.	Walsh	Zionsville, IN
Donald J.	Merlo	Carmel, IN

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max):

Novel Herbicide Resistance Genes

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☒ Customer Number 23557

OR

☐ Firm or Individual Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS ***(check all that apply)***

☐ Application Data Sheet. See 37 CFR 1.76
☒ Specification *Number of Pages* 117
☒ Drawing(s) *Number of Sheets* 1
☐ CD(s), Number of CDs _____
☒ Other (specify) Sequence Listing (3 pages)

Fees Due: Filing Fee of $200 ($100 for small entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is due, which is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

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☐ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fee and application size fee (if applicable).
☐ Payment by credit card. Form PTO-2038 is attached.

TOTAL FEE AMOUNT ($)
$450.00

☒ The Director is hereby authorized to charge the filing fee and application size fee (if applicable) or credit any payment to Deposit Account Number: 19-0065 . A duplicative copy of this form is enclosed for fee processing.

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

SIGNATURE _____

Date October 28, 2005

REGISTRATION NO. 39,355

TYPED or PRINTED NAME Jay M. Sanders

Docket Number: DAS-129P

TELEPHONE (352) 375-8100

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This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. : DAS-129P

Applicant(s) : Terry R. Wright, Justin M. Lira, Terry Walsh, Donald J. Merlo

For : Novel Herbicide Resistance Genes

COMMISSIONER FOR PATENTS
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Express Mail No.. EQ005884833US Date of Deposit: October 28, 2005

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Drew Core
Typed/Printed Name of Person Mailing Paper

Signature

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NOVEL HERBICIDE RESISTANCE GENES

Background of the Invention

[0001] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0002] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

[0003] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (*e.g.*, wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (*e.g.*, alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; *see* "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0004] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

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[0005] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Ng *et al.*, 2003; Simarmata *et al.*, 2003; Lorraine-Colwill *et al.*, 2003; Sfiligoj, 2004; Miller *et al.*, 2003; Heap, 2005; Murphy *et al.*, 2002; Martin *et al.*, 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis,* and *Plantago lanceolata.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0006] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

[0007] 2,4-D is in the phenoxy acid class of herbicides, as is MCPA. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf

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weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known. Triclopyr and fluroxypyr are pyridyloxyacetic acid herbicides whose mode of action is as a synthetic auxin, also.

[0008] These herbicides have different levels of selectivity on certain plants (*e.g.*, dicots are more sensitive than grasses). Differential metabolism by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

[0009] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D currently formulated has a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0010] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0011] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-

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ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0012] One unique example with low homology to *tfdA* (31% amino acid identity) is *sdpA* from *Delftia acidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002, Westendorf *et al., 2003*). This enzyme has been shown to catalyze the first step in (S)-dichlorprop (and other (S)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Transformation of this gene into plants, has not heretofore been reported.

[0013] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0014] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxyacetate auxins (such as 2,4-D and dichlorprop), pyridyloxyacetate auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. A multifunctional enzyme for the degradation of herbicides covering multiple modes of action has recently been described (PCT US/2005/014737; filed May 2, 2005). Another unique multifunctional enzyme and potential uses are described hereafter.

Brief Summary of the Invention

[0015] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, aryloxyalkanoate-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more

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robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0016] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, MCPA, triclopyr, fluroxypyr, and one or more commercially available herbicides (*e.g.*, glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0017] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide-tolerant crop (HTC) trait and selectable marker opportunities.

[0018] There was no prior motivation to produce plants comprising an *AAD-12* gene (preferably an *AAD-12* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-12 enzyme to render the plants resistant to not only phenoxyacetic acid herbicides (such as 2,4-D) but also the pyridyloxyacetates herbicides,

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triclopyr and fluroxypyr. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0019] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxyacetate auxin and pyridyloxyacetates auxin herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid and other aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more pyridyloxyacetate or phenoxyacetate auxin herbicides to plants comprising an *AAD-12* gene. The subject invention also provides methods of using an *AAD-12* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-12*, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0020] **Figure 1** illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop.

Brief Description of the Sequences

[0021] **SEQ ID NO:1** is the nucleotide sequence of *AAD-12* from *Delftia acidovorans*.

[0022] **SEQ ID NO:2** is the translated protein sequence encoded by SEQ ID NO:1.

[0023] **SEQ ID NO:3** is the plant optimized nucleotide sequence of *AAD-12 (v1)*.

[0024] **SEQ ID NO:4** is the translated protein sequence encoded by SEQ ID NO:3.

[0025] **SEQ ID NO:5** is the E.coli optimized nucleotide sequence of *AAD-12 (v2)*.

[0026] **SEQ ID NO:6** is the sequence of the M13 forward primer.

[0027] **SEQ ID NO:7** is the sequence of the M13 reverse primer.

[0028] **SEQ ID NO:8** is the sequence of the forward PTU primer.

[0029] **SEQ ID NO:9** is the sequence of the reverse PTU primer.

[0030] **SEQ ID NO:10** is the sequence of the *AAD-12 (v1)* PTU forward PCR primer.

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[0031] **SEQ ID NO:11** is the sequence of the *AAD-12 (v1)* PTU reverse PCR primer.

[0032] **SEQ ID NO:12** shows the sequence of the "NcoI of Brady" primer.

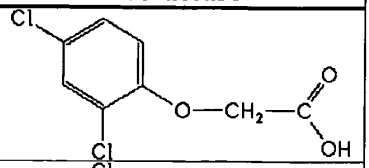
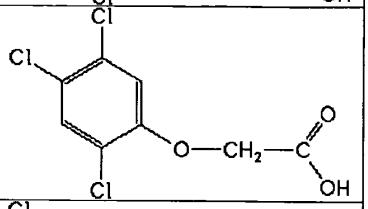
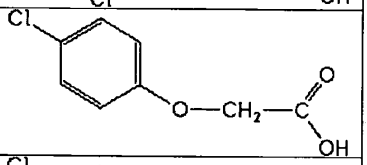
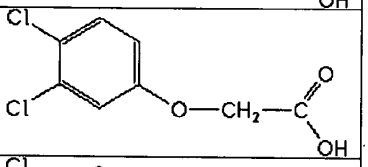
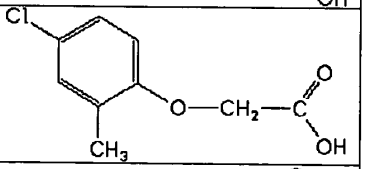
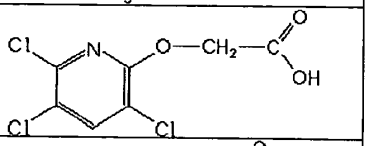
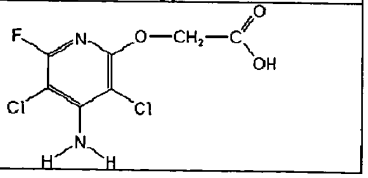
[0033] **SEQ ID NO:13** shows the sequence of the "SacI of Brady" primer.

Detailed Description of the Invention

[0034] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the *AAD-12* gene is that unlike all *tfdA* homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (*e.g.,* triclopyr, fluroxypyr) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). *See* **Table 1**. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the *AAD-12* gene in plants would afford protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled, protecting from drift or other off-site synthetic auxin herbicide injury for the full breadth of commercially available phenoxy auxins. **Table 1** defines commercially available pyridyl and phenoxy auxins and provides relevant chemical structures.

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Table 1. Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	
MCPA	94-74-6	25 – 4000	125 - 1550	
Triclopyr	55335-06-3	50 – 2000	70 – 840	
Fluroxypyr	69377-81-7	25 – 2000	35 – 560	

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[0035] A single gene (*AAD-12*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-12* can provide protection *in planta* to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing *AAD-12* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

[0036] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-12* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (*e.g.*, 2,4-D) and pyridyloxyacetates auxin herbicides (*e.g.*, triclopyr)—selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0037] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the

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ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/*AAD-12* stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0038] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0039] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS, GOX, GAT*), glufosinate resistance (*e.g.*, *Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g.*, *PPO-1*), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), dicamba-degrading enzymes (*see, e.g.*, US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

[0040] Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione,

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isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0041] Additionally, *AAD-12* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0042] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-1 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

[0043] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also pyridyloxyacetate herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0044] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and pyridyloxyacetate herbicides. Thus, the subject invention relates in part to the degradation of

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2,4-dichlorophenoxyacetic acid, other phenoxyacetic acids, and pyridyloxyacetic acid herbicides by a recombinantly expressed AAD-12 enzyme.

[0045] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP *in vitro*. An additional advantage that *AAD-12* transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0046] The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an *AAD-12* gene.

[0047] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0048] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0049] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-

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ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0050] The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, *sdpA*, from *Delftia acidivorans* (Westendorf *et al.*, 2002, 2003) with low homology to *tfdA* (31% amino acid identity). This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. *SdpA* has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0051] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

[0052] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and pyridyloxy auxin herbicides.

[0053] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. *AAD-12* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (*e.g.*, glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, *et al.*), and insect resistance traits (*Cry1F, Cry1Ab, Cry 34/45, et al.*) for example.

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Additionally, *AAD-12* can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0054] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-12*-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention.

[0055] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[0056] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

[0057] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[0058] The transgenic expression of the subject *AAD-12* genes is exemplified in, for example, *Arabidopsis*, tobacco, soybean, and canola. However, the subject invention can be applied to any other desired types of plants, such as corn (maize), cotton, and rice. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as warm season turf grass) and dicot crops. Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in

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grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

[0059] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

[0060] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

[0061] Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that

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the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[0062] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

[0063] The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[0064] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[0065] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as

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genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the order Proteobacteria, and specific genera with known *sdpA*'s, such as *Sphingobium* and *Delftia*, for example, can be used as source isolates.

[0066] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[0067] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[0068] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook *et al.*, 1989, which is incorporated herein by reference.

[0069] Polynucleotides and probes. The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[0070] The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control

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of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-12* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

[0071] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[0072] In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

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[0073] Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

[0074] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

[0075] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl

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(175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[0076] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[0077] Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

[0078] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

$$Tm = 81.5° C + 16.6 \text{ Log}[Na+] + 0.41(\%G+C) - 0.61(\%\text{formamide}) - 600/\text{length of duplex in base pairs.}$$

[0079] Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

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[0080] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

Tm (°C) = 2(number T/A base pairs) + 4(number G/C base pairs)

(Suggs *et al.*, 1981).

[0081] Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

[0082] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low:	1 or 2x SSPE, room temperature
Low:	1 or 2x SSPE, 42° C
Moderate:	0.2x or 1x SSPE, 65° C
High:	0.1x SSPE, 65° C.

[0083] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[0084] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.*, 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the

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primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[0085] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[0086] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be

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made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[0087] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[0088] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[0089] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226,

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227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, or 293 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[0090] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[0091] It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.*, Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term "protein" can include functionally active truncations.

[0092] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62,

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63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[0093] Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[0094] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.*, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[0095] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within

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the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 2** provides a listing of examples of amino acids belonging to each class.

Table 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[0096] In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

[0097] As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

[0098] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

[0099] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or

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monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

[00100] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[00101] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[00102] Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[00103] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[00104] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression

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of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[00105] Vectors comprising an *AAD-12* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc.* Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli.* The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

[00106] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria.* The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria.*

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They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[00107] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00108] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[00109] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (*PAT*), glyphosate (*EPSPS*), imazethyapyr (*AHAS*), and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins,

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thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00110] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. *See* U.S. Patent No. 6,809,232. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00111] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the

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construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[00112] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00113] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

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[00114] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00115] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

[00116] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00117] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like

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may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00118] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00119] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are

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described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00120] Unless specifically indicated or implied, the terms "a", "an", and "the" signify "at least one" as used herein.

[00121] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00122] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Method for Identifying Genes That Impart Resistance to 2,4-D *In Planta*

[00123] As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (*i.e.*, α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with ≤ 50% homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to *tfdA* from *Ralstonia eutropha)* can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00124] A single gene (*sdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 31% amino acid

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identity to *tfdA*. Percent identity was determined by first translating both the *sdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

Example 2 – Optimization of Sequence for Expression in Plants and Bacteria

[00125] 2.1 – Background.

[00126] To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[00127] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00128] **Table 3** illustrates how high the G+C content is in maize. For the data in **Table 3**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

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Table 3: Compilation of G + C contents of protein coding regions of maize genes

Protein Class[a]	Range % G + C	Mean % G + C[b]
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)[c]

[a] Number of genes in class given in parentheses.
[b]Standard deviations given in parentheses.
[c] Combined groups mean ignored in mean calculation

[00129] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[00130] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 4**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to

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specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, *etc.* with the next preferred codon of choice.

Table 4. Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

[00131] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first

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choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00132] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

[00133] 2.2 – *AAD-12* Plant rebuild analysis.

[00134] Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native *AAD-12* coding region (SEQ ID NO:1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 (AAD-12) is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence *AAD-12 (v1)* (SEQ ID NO:3) was developed that encodes a protein (SEQ ID NO:4) which is the same as the native SEQ ID NO:2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO:4). The additional alanine codon (GCT; underlined in SEQ ID NO:3) encodes part of an *Nco*I restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical. **Table 5** shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences

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(Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

Table 5. Codon composition comparisons of coding regions of Native AAD-*12*, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #	Amino Acid	Codon	Native #	Plant Opt v1 #	Theor. Plant Opt. #
ALA (A)	GCA	1	10	11	LEU (L)	CTA	0	0	0
	GCC	35	16	15		CTC	1	8	8
	GCG	7	0	0		CTG	23	0	0
	GCT	0	18	17		CTT	0	8	8
ARG (R)	AGA	0	4	5		TTA	0	0	0
	AGG	0	4	6		TTG	0	8	8
	CGA	0	0	0	LYS (K)	AAA	1	1	2
	CGC	15	6	4		AAG	5	5	4
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	4	3	PHE (F)	TTC	7	5	5
ASN (N)	AAC	3	2	2		TTT	1	3	3
	AAT	1	2	2	PRO (P)	CCA	0	5	6
ASP (D)	GAC	15	9	9		CCC	9	4	4
	GAT	2	8	8		CCG	5	0	0
CYS (C)	TGC	3	2	2		CCT	0	5	5
	TGT	0	1	1	SER (S)	AGC	5	4	3
END	TAA	1	0	1		AGT	0	0	0
	TAG	0	0			TCA	0	3	3
	TGA	0	1			TCC	2	3	3
GLN (Q)	CAA	1	8	7		TCG	6	0	0
	CAG	13	6	7		TCT	0	3	3
GLU (E)	GAA	3	4	4	THR (T)	ACA	1	4	5
	GAG	8	7	7		ACC	11	7	7
GLY (G)	GGA	0	8	7		ACG	5	0	0
	GGC	24	7	7		ACT	1	7	6
	GGG	1	3	4	TRP (W)	TGG	8	8	8
	GGT	0	7	7	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	9	9		TAT	1	2	2
	CAT	8	7	7	VAL (V)	GTA	0	0	0
ILE (I)	ATA	0	2	2		GTC	6	8	7
	ATC	10	4	5		GTG	18	8	9
	ATT	1	5	4		GTT	0	8	8
	Totals	163	164	163		Totals	130	130	130

[00135] It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

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[00136] 2.3 Rebuild for *E. coli* Expression

[00137] Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in *Escherichia coli and Salmonella typhimurium cellular and molecular biology, vol. 2*, pp. 2047–2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umbarger, H. (eds.) American Society for Microbiology, Washington, DC). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for these *E. coli*-Class II gene coding regions. It is thought that a protein coding region having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO:4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (*i.e.* Shine Dalgarno sequences) The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO:5 and encodes the protein disclosed in SEQ ID NO:4.

[00138] The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. **Table 6A** presents the codon compositions of the native *AAD-12* coding region (Columns A and D), an *AAD-12* coding region optimized for expression in *E. coli* (v3; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).

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Table 6A. Codon composition comparisons of coding regions of Native *AAD-12*, *E. coli*-Optimized version (v2) and a Theoretical *E. coli* Class II-Optimized version.

		A	B	C			D	E	F
Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor. Class II #	Amino Acid	Codon	Native #	*E. coli* Opt v2 #	Theor, Class II #
ALA (A)	GCA	1	13	13	LEU (L)	CTA	0	0	0
	GCC	35	0	0		CTC	1	2	0
	GCG	7	18	17		CTG	23	20	24
	GCT	0	13	14		CTT	0	1	0
ARG (R)	AGA	0	0	0		TTA	0	1	0
	AGG	0	0	0		TTG	0	0	0
	CGA	0	0	0	LYS (K)	AAA	1	4	5
	CGC	15	6	6		AAG	5	2	1
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	12	12	PHE (F)	TTC	7	6	6
ASN (N)	AAC	3	4	4		TTT	1	2	2
	AAT	1	0	0	PRO (P)	CCA	0	3	2
ASP (D)	GAC	15	10	9		CCC	9	0	0
	GAT	2	7	8		CCG	5	11	12
CYS (C)	TGC	3	2	2		CCT	0	0	0
	TGT	0	1	1	SER (S)	AGC	5	4	4
END	TAA	1	1	1		AGT	0	0	0
	TAG	0	0	0		TCA	0	0	0
	TGA	0	0	0		TCC	2	5	4
GLN (Q)	CAA	1	3	3		TCG	6	0	0
	CAG	13	11	11		TCT	0	4	5
GLU (E)	GAA	3	8	8	THR (T)	ACA	1	0	0
	GAG	8	3	3		ACC	11	12	12
GLY (G)	GGA	0	0	0		ACG	5	0	0
	GGC	24	12	11		ACT	1	6	6
	GGG	1	0	0	TRP (W)	TGG	8	8	8
	GGT	0	13	14	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	11	11		TAT	1	2	2
	CAT	8	5	5	VAL (V)	GTA	0	6	6
ILE (I)	ATA	0	0	0		GTC	6	0	0
	ATC	10	7	7		GTG	18	8	7
	ATT	1	4	4		GTT	0	10	11
	Totals	163	164	164		Totals	130	130	130

[00139] It is clear from examination of **Table 6A** that the native and *E. coli*-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

[00140] 2.4 – Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence

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of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO:20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00141] A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in **Table 6B**. Columns D and H of **Table 6B** present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in **Table 6B.** Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

Weighted % of C1 =1/(%C1 + %C2 + %C3 + *etc.*) x %C1 x 100

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of **Table 6B** (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of **Table 6B**. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

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Table 6B. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	Codon	Weighted %	Soybean %	Amino Acid	Codon	Weighted %	Soybean %
ALA (A)	GCA	33.1	30.3	LEU (L)	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	LYS (K)	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	**6.0**	MET (M)	ATG	100.0	100
	CGT	16.9	14.5	PHE (F)	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	PRO (P)	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	SER (S)	AGC	16.0	15.1
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	**6.1**
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	THR (T)	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	**8.3**
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	TRP (W)	TGG	100.0	100
	GGT	30.4	30.4	TYR (Y)	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	VAL (V)	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

[00142] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 from PCT/US2005/014737 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the

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numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21 of PCT/US2005/014737.

[00143] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is disclosed as bases 1-1575 of SEQ ID NO:22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO:22 of PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston TX).

Example 3-Cloning of expression and transformation vectors

[00144] 3.1 Construction of E.coli, pET expression vector.

[00145] Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba 1, Xho 1) *AAD-12 (v2)* was cut out of the picoscript vector X and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

[00146] To differentiate between *AAD-12 (v2)*:pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C for 4 hours with agitation. Each culture was then spotted onto LB K plates, which were incubated at 37° C overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3222.

[00147] 3.2- Construction of *Pseudomonas* expression vector

[00148] The *AAD-12 (v2)* open reading frame was initially cloned into the modified pET expression vector (Novagen), "pET280 S/S", as an XbaI – XhoI fragment. The resulting

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plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The AAD12 open reading frame from pDAB725 was transferred into the *Pseudomonas* expression vector, pMYC1803, as an XbaI – XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB321 *Pseudomonas* expression strains.

[00149] 3.3 – Completion of binary vectors.

[00150] The plant optimized gene *AAD-12 (v1)* was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:6) and M13 Reverse (SEQ ID NO:7) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-12 (v1)* DNA sequence. The *AAD-12 (v1)* gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-12 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB724, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-12 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium* (see section 7.2).

[00151] 3.4 - Cloning of additional transformation constructs.

[00152] All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular cloning methods (Maniatis *et al.,* 1982). **Table 7** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

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Table 7. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
724		A, Ct, S	AAD12 v1	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3274		A	AAD12 v1	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	-	-	Agro binary
3277		S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2			Spectinomycin		pat	AtUbi10	Agro binary
3278	1580	T	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2			Spectinomycin	-	pat	AtUbi10	Agro binary
3285		A	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3759	-	A, Ca, S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi10	Agro binary
4101		Cn, R	AAD12 v1	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers / Gun

*A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region

(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

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Example 4 - Recombinant AAD-12 (v2) Expression in *Pseudomonas fluorescens*

[00153] 4.1- *Pseudomonas fluorescens* culture

[00154] 200 µl of *Pseudomonas fluorescens* glycerol stock carried plant-optimized *AAD-12* gene (v2) was used to inoculate 50 ml fresh LB media supplemented with 30 µg/ml tetracycline/HCl. The culture (in a 250 ml baffled Erlenmeyer flask) was incubated on a shaker (New Brunswick Scientific Model Innova 44) at 300 rpm and 30 °C for 16 hrs. 20 ml of seed culture was transferred into 1 L *Pseudomonas fluorescens* culture media (Yeast extract, 5 g/L; K_2HPO_4, 5 g/L; $(NH_4)_2PO_4$, 7.5 g/L; $(NH_4)_2SO_4$; $MgSO_4$-$7H_2O$, 1 g/L; KCl, 0.5 g/L; $CaCl_2$-$2H_2O$, 0.5 g/L; NaCitrate-$2H_2O$, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: $FeCl_3$-$6H_2O$, 5.4 g/L; $MnCl_2$-$4H_2O$, 1 g/L; $ZnSO_4$-$7H_2O$, 1.45 g/L; $CuSO_4$-$5H_2O$, 0.25 g/L; H_3BO_3, 0.1 g/L; $(NH_4)_6MO_7O_{24}$, 0.1 g/L; concentrated HCl, 13 ml/L) supplemented with 20 µg/ml tetracycline/HCl and 250 µl of anti-form Pluronic L61 in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 30°C and 300 rpm for 24 hrs. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM final in the cultures and continued to incubate for 48 hrs at same conditions. Cells were harvested by centrifugation at 7 krpm at 4 °C for 15 min, and cell paste was stored at -80 °C or immediately processed for purification.

[00155] 4.2- Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

[00156] Frozen or fresh *Pseudomonas* cells from 6 L culture were thawed and resuspended in approximately 1000 ml of extraction buffer containing 20 mM Tris-HCl, pH 8.5, and 25 ml of Protease Inhibitor Cocktail (Sigma cat# P8465), and disrupted on ice for 20 minutes by sonication. The lysate was centrifuged at 24,000 RPM for 20 min. The supernatant was transferred, and dialyzed against 5 L of 20 mM Tris-HCl, pH 8.5 overnight at 4 °C, and applied at 15 ml/min to a Q Sepharose Fast Flow column (Pharmacia XK 50/00, 500 ml bed size) equilibrated with the same buffer. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4 °C. The column was washed with this buffer until the eluate OD_{280} returned to baseline, then proteins were eluted with 2 L of linear gradient from 0 to 0.2 M NaCl at a flow rate of 15 ml/min, while 45 ml fractions were collected. Fractions containing AAD-12 activity as determined by the colorimetric enzyme assay and also corresponding to the predicted molecular weight of AAD-12 protein (about 32 kDa band on SDS-PAGE) were pooled. Solid ammonium sulfate to final 0.5 M was added to

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the sample, and then applied to a Phenyl Source 15 column (Pharmacia HR 16/10, 20 ml bed size) equilibrated in 0.5 M ammonium sulfate in 20 mM Tris-HCl, pH 8.5. This column was washed with the binding buffer at 4 ml/min until the OD_{280} of the eluate returned to baseline, then proteins were eluted within 50 min (4 ml/min) by a linear gradient from 0.5 M to 0 Ammonium sulfate in 20 mM Tris-HCl, pH 8.5, and 4 ml fractions were collected. The main peak fractions contain AAD-12 was pooled, concentrated to 5 ml using MWCO 10 kDa membrame centrifugal filter device (Millipore). The sample was then applied to a Superdex 75 gel filtration column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure AAD-12 were pooled and stored at -80 °C. Protein concentration was determined by Bradford assay or total amino acid analysis using bovine serum albumin as standard.

[00157] Ten milligrams purified AAD-12 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

Example 5 – *In vitro* assays of AAD-12 activity

[00158] 5.1 – Assay via colorimetric phenol detection.

[00159] Enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 µM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 µM $NH_4(FeSO_4)_2$, 200 µM sodium ascorbate. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol was linear and proportional to the concentration of phenol in the assay up to ~500 µM. A calibration curve

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performed under standard assay conditions (160 µl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2 µM phenol.

[00160] Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200 µM NH_4FeSO_4, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 5 minutes of incubation at 25° C, the reaction was terminated by addition of 30 µl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH) and 0.2 % 4-aminoantipyrine. Then 10 µl 0.8 % potassium ferricyanide was added and after 5 or 10 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols.

[00161] 5.2 – Assay via detection of chloropyridinol

[00162] AAD-12 action on potential substrates such as the herbicide triclopyr containing a substituted pyridine (rather than benzene rings) will release a pyridinol on cleavage of the aryloxyalkanoate bond. Pyridinols were not detected using the aminoantipyrine/ferricyanide phenol detection described in the preceding section. However, it was found that product chloropyridinols absorb strongly in the near UV with λ_{max} of 325 nm at pH 7 (extinction coefficient ~8,400 $M^{-1}.cm^{-1}$). This was used to create a continuous microplate-based spectrophotometric assay. Assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 µM NH_4FeSO_4, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero and the increase in absorbance followed for 10 minutes at 325 nm in a microplate reader. The first 2 minutes of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 µl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9 µM chloropyridinol.

[00163] 5.3 – Colorimetric assay using 2-(2-chloro,4-nitrophenoxy)propionate

[00164] A convenient assay of AAD-12 was devised using 2-(2-chloro,4-nitrophenoxy)propionate (CNPP) as substrate. Cleavage of CNPP by AAD-12 releases 2-chloro,4-nitrophenol. This phenol has a bright yellow absorbance at 410 nm at pH 7 enabling the reaction to be followed continuously or by endpoint analysis. The presence of AAD-12

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activity can be monitored visually without the need for addition of further reagents. Microplate-based spectrophotometric assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate amount of CNPP (added from a 10 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of CNPP, enzyme, or α-ketoglutarate at time zero and the increase in absorbance followed for 10 min at 410 nm in a microplate reader. The first 2 min of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200 μl final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1 μM 2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be $K_m = 31 \pm 5.5$ μM and $k_{cat} = 16.2 \pm 0.79$ min^{-1}.

Example 6 – In vitro activity of AAD-12 on various substrates

[00165] 6.1 – AAD-12 (v2) activity on (R,S)- dichlorprop, (R)-dichlorprop, (S)- dichlorprop and 2,4-D

[00166] Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4 μg purified AAD-12 (v2). (R,S)-dichlorprop (R,S-DCP) was tested at 1 mM and (R)-dichlorprop, (S)-dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in Figure 1, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4 μg AAD-12 (v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of α-ketoglutarate. After 5 minutes, the reaction was quenched, and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

[00167] AAD-12 (v2) has excellent activity on (R,S)-dichlorprop and (S)-dichlorprop and has minimal activity on (R)-dichlorprop. This indicates that AAD-12 (v2) has a clear (S)-enantiomeric preference. The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)-dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively.

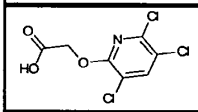
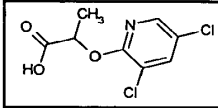
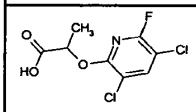
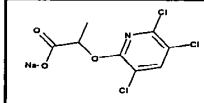
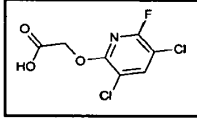
[00168] 6.2 – AAD-12 (v2) activity on pyridyloxyalkanoates

[00169] Using the pyridinol assay described in Example 5.2, five pyridyloxyalkanoates were assayed at 1 mM in a reaction mix containing 6.8 μg purified AAD-12 (v2). The rates of each

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reaction were monitored and are presented in **Table 8**. All five pyridyl-oxyalkanoates were cleaved to release pyridinols by AAD-12 (v2). The rates for the oxypropionate substrates 116844 and 91767 were somewhat faster than those for the corresponding acetates (triclopyr and 93833 respectively) indicating a preference of AAD-12 (v2) for oxypropionate over oxyacetate side chains. These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.

Table 8: Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8 µg AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of α-ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without α-ketoglutarate was subtracted from the rates with substrate

STRUCTURE	ID	Rate (mAU/min)	Rate relative to triclopyr
	triclopyr	97	1
	66357	225	2.3
	91767	190	0.8
	116844	257	1.4
	93833	118	0.5

[00170] 6.3 – Kinetic constants of AAD-12 (v2) for 2,4-D, (R,S)-DCP and triclopyr

[00171] The K_m and k_{cat} values of purified AAD-12 (v2) for the herbicides 2,4-D, (R,S)-dichlorprop and triclopyr were determined using the appropriate assay method. Substrate inhibition occurred at high concentrations (>1mM) of 2,4-D and (R,S)-DCP so concentrations below this were used to fit the data to the Michaelis-Menten equation using Grafit 4.0

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(Erithacus Software, UK). No substrate inhibition was noted for triclopyr up to 2 mM. The kinetic constants are summarized in **Table 9**. From these data, the rate of AAD-12 (v2) cleavage of triclopyr is ~5% that of 2,4-D, under maximal velocity conditions.

Table 9: Kinetic constants of AAD-12 (v2) for three herbicide substrates

Substrate	K_m, µM (±SE)	k_{cat}, min^{-1} (±SE)	Assay method	Substrate inhibition at 2 mM
2,4-D	102 (±18.4)	54.1 (±3.1)	Phenol detection	55%
(R,S)-dichlorprop	122 (±2.7)*	63.4 (±0.5)	Phenol detection	55%
Triclopyr	241 (±30)	2.6 (±0.1)	ΔA325 nm	0%

* Because of the (S)-enantiomeric preference of AAD-12, the K_m value was calculated assuming 50% of the racemic mixture was available as a substrate

Example 7 – Transformation into *Arabidopsis* and Selection

[00172] 7.1 – *Arabidopsis thaliana* growth conditions.

[00173] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00174] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00175] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 µmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

[00176] 7.2 – *Agrobacterium* transformation.

[00177] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

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[00178] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 µl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00179] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00180] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed Agrobacterium cells, suspended in 10 µl of water, at 100° C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

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[00181] 7.3 – *Arabidopsis* transformation.

[00182] *Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044μM benzylamino purine (10 μl/liter of 1 mg/ml stock in DMSO) and 300 μl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

[00183] 7.4 – Selection of transformed plants.

[00184] Freshly harvested T_1 seed [*AAD-12 (v1)* gene] was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00185] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00186] Seven days after planting (DAP) and again 11 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703

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L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-12 (v1)* (plant optimized gene) to provide phenoxy auxin herbicide resistance.

[00187] Random T_1 plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis* 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200g ae/ha). Tables 10 and 11 show comparisons drawn to an aryloxyalkanoate herbicide resistance gene (*AAD-1 v3*) previously described in PCT/US2005/014737.

[00188] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

[00189] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluroxypyr, could be effectively degraded *in planta*. Herbicides were applied to T_1 plants with use of a track sparyer in a 187 L/ha spray volume. T_1 plants that exhibited tolerance to 2,4-D DMA were further accessed in the T_2 generation.

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[00190] 7.5 – Results of selection of transformed plants.

[00191] The first *Arabidopsis* transformations were conducted using *AAD-12 (v1)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T_1 seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT + Liberty are used for selection. T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 10** compares the response of *AAD-12 (v1)* and control genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The *AAD-12 (v1)* gene imparted herbicide resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 10** simply to demonstrate the significant difference between the plants transformed with *AAD-12 (v1)* versus the wildtype or *PAT/Cry1F*-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or 6x field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

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Table 10. *AAD-12 v1* (plant optimized)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence compared to or *AAD-1 v3* (T_4) homozygous resistant population, Pat-Cry1F transformed, auxin-sensitive control.

AAD-12 v1 gene T_1 transformants	% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control-buffer	6	0	0	0	0
50 g ae/ha 2,4-D	6	0	2	16	24
200 g ae/ha 2,4-D	6	1	1	11	18
800 g ae/ha 2,4-D	5	2	1	15	20
3200 g ae/ha 2,4-D	8	0	0	6	6
PAT/Cry1F (transformed control)	% Injury			% Injury Ave	Std Dev
Averages	<20%	20-40%	>40%		
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	4	1	5	31	16
200 g ae/ha 2,4-D	0	0	10	70	2
800 g ae/ha 2,4-D	0	0	10	81	8
3200 g ae/ha 2,4-D	0	0	10	91	2
Homozygous AAD-1 (v3) gene T_4 plants	% Injury			% Injury Ave	Std Dev
Averages	<20%	20-40%	>40%		
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	10	0	0	0	0
200 g ae/ha 2,4-D	10	0	0	0	0
800 g ae/ha 2,4-D	10	0	0	0	0
3200g ae/ha 2,4-D	9	1	0	2	6

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Table 11 shows a similarly conducted dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. The data shows that the herbicidally active (R-isomer of diclorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-diclorprop is one distinguishing characteristic that separates the two genes. (**Table 11**). AAD-1 and AAD-12 are considered R- and S-specific α-ketoglutarate dioxygenases, respectively.

Table 11. T_1 *Arabidopsis* response to a range of R-dichlorprop rates applied postemergence.

AAD-12 v1 gene	% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control	6	0	0	0	0
50 g ae/ha R-dichlorprop	0	0	8	63	7
200 g ae/ha R-dichlorprop	0	0	8	85	10
800 g ae/ha R-dichlorprop	0	0	8	96	4
3200 g ae/ha R-dichlorprop	0	0	8	98	2
PAT/Cry1F	% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	0	10	0	27	2
200 g ae/ha R-dichlorprop	0	0	10	69	3
800 g ae/ha R-dichlorprop	0	0	10	83	6
3200 g ae/ha R-dichlorprop	0	0	10	90	2
Homozygous AAD-1 (v3) gene	% Injury			% Injury	
T_4 plants	<20%	20-40%	>40%	Ave	Std Dev
Untreated control	10	0	0	0	0
50g ae/ha R-dichlorprop	10	0	0	0	0
200 g ae/ha R-dichlorprop	10	0	0	0	0
800 g ae/ha R-dichlorprop	10	0	0	0	0
3200 g ae/ha R-dichlorprop	10	0	0	0	0

[00192] 7.6 – *AAD-12 (v1)* as a selectable marker:

[00193] The ability to use *AAD-12 (v1)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. Approximately 50 T_4 generation Arabidopsis seed (homozygous for AAD-12 (v1)) was spiked into approximately 5,000 wildtype (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 follwed by 11 DAP. Since all individuals also contained the PAT gene comparison concomitant with the transformtation vetor, comparison of AAD-12 / 2,4-D selected and glufosinate selection was made.

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Treatments were applied with a DeVilbiss spray tip as previously describd. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate AAD-12 can effectively be used as an alternative selectable marker for a population.

[00194] 7.7 – Heritability.

[00195] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 55% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00196] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show *AAD12 (v1)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

[00197] 7.8 – Additional foliar applications herbicide resistance in AAD-12 *Arabidopsis*.

[00198] The ability of *AAD-12 (v1)* to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates. T_2 generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing *PAT* and the insect resistance gene *Cry1F* was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Dow AgroSciences) and 280-2240 g ae/ha fluroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ae/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

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[00199] These results (see **Table 12**) confirm that *AAD-12 (v1)* in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on transgenic non-AAD-12 control *Arabidopsis (Pat/Cry1F)*. This is the first report of an enzyme with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

Table 12. Comparison of T_2 AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

	Pyridyloxyacetic auxins	
	Ave % Injury 14DAT	
Herbicide Treatment	**Segregating T_2 *AAD-12 (v1)*plants (pDAB724.01.120)**	**Pat/Cry1f - Control**
280 g ae/ha Triclopyr	0	52
560 g ae/ha Triclopyr	3	58
1120 g ae/ha Triclopyr	0	75*
2240 g ae/ha Triclopyr	3	75*
280 g ae/ha Fluroxypyr	0	75*
560 g ae/ha Fluroxypyr	2	75*
1120 g ae/ha Fluroxypyr	3	75*
2240 g ae/ha Fluroxypyr	5	75*
	Inactive DCP metabolite	
280 g ae/ha 2,4-DCP	0	0
560 g ae/ha 2,4-DCP	0	0
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

* Plants in this experiment were stunted and severely epinastic, but remained green and did not receive injury ratings > 75%.

[00200] 7.9 – Molecular analysis of *AAD-12 (v1) Arabidopsis*.

[00201] Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-12 (v1)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-12 (v1)*. Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00202] Results showed all 2,4-D resistant plants assayed contained *PAT* (and thus by inference, *AAD-12 (v1)*). Copy number analysis showed total inserts ranged from 1 to 5

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copies. This correlates, too, with the *AAD-12 (v1)* protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

[00203] 7.10 – *Arabidopsis* transformed with molecular stack of *AAD-12 (v1)* and glyphosate resistance gene.

[00204] T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3759 plasmid (*AAD-12 (v1)* + *EPSPS*) coding for a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-12 (v1)* as the selectable marker as described in example 7.6. T_1 plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-12 (v1)* + *PAT* T_4 homozygous lines (pDAB724-transformed), and *PAT* + *Cry1F* homozygous line (transformed control). pDAB3759 and pDAB724 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

[00205] 7.11 – *AAD-12 Arabidopsis* genetically stacked with *AAD-1* to give wider spectrum of herbicide tolerance.

[00206] *AAD-12 (v1)* (pDAB724) and *AAD-1 (v3)* (pDAB721) plants were reciprocally crossed and F_1 seed was collected. Eight F_1 seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F_1 plants and subjected to Western analysis to confirm the presence of both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

[00207] One hundred F_2 seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F_2 plants survived glufosinate selection fitting an expected segregation ration for two independently assorting loci for glufosinate resistance (15 R : 1S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop + 560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are aconsistent with an expected segregation pattern (9R:

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6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlroprop or triclopyr). The results indicate that *AAD-12* (pDAB724) can be successfully stacked with *AAD-1* (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D + R-dichlorprop) and (2,4-D + fluroxypyr + triclopyr), respectively]. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F1 generation through paired sprays with herbicides that are exclusive between the AAD-1 and AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively, above).

Example 8 –WHISKERS-mediated transformation using imazethapyr selection

[00208] 8.1 – Cloning of *AAD-12 (v1)*.

[00209] The *AAD-12 (v1)* gene was cut out of the intermediate vector pDAB3283 as an NcoI/SacI fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1 : *AAD-12 (v1)* cassette. This construct was digested with NotI and PvuI to liberate the gene cassette and digest the unwanted backbone. This was ligated to NotI cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101, which contains ZmUbi1/*AAD-12 (v1)* / ZmPer5::OsAct1/AHAS /ZmLip.

[00210] 8.2 – Callus/suspension initiation.

[00211] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

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[00212] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00213] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

[00214] 8.3 – Cryopreservation and thawing of suspensions.

[00215] Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes

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within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

[00216] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

[00217] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00218] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After

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agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00219] Approximately 2mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

[00220] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 3 μM imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8) Plates were placed in boxes and cultured for an additional week.

[00221] Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C) containing 3 μM imazethapyr. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C in dark boxes.

[00222] 8.4- Protocol for Plant Regeneration. Putatively transformed isolates are typically first visible 5-8 weeks post-transformation. Any potential isolates are removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained growth is evident after approximately 2 weeks, an event is deemed to be resistant and is submitted for molecular analysis.

[00223] Regeneration is initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3 μM imazethapyr, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells are allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week, then higher light (40 $\mu Em^{-2}s^{-1}$) for another week, before being transferred to regeneration medium, 36 (3P), which is identical to 28 (3P) except that it lacks plant growth regulators. Small (3-5 cm) plantlets are removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once

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plantlets developed a sufficient root and shoot system, they are transplanted to soil in the greenhouse.

[00224] 8.5– Molecular Analysis: Maize Materials and Methods.

[00225] 8.5.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00226] 8.5.2 – Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl is placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well is overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

[00227] 8.5.3 – Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-12 (v1)* PTU are (Forward – ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:8) and (Reverse – CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR *AAD-12 (v1)* are (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:10) and (Reverse – CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute

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and 45 seconds followed by 72° C for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00228] 8.5.4 – Southern blot analysis. Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 μg of plasmid DNA is subjected to an overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85 % agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

Example 9. Protein detection from transformed plants via antibody

[00229] 9.1 – Extracting AAD-12 (v1) from plant leaves.

[00230] Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 μL plant extraction buffer (PBS containing 0.1 % Triton X-100 and 10 mM DTT). The tubes were shaken for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000x g. The supernatant containing the plant soluble proteins

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were analyzed for both total soluble protein (TSP) and AAD-12 (v1) using Western Blots and ELISA.

[00231] 9.2 – Bradford assay.

[00232] Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five microliters of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For BSA standards, concentrations were 2000, 1500, 1000, 750, 500, 250 and 125 μg/mL. The Protein Assay Concentrate (BioRad, Hercules, CA) was first diluted 5 fold in PBS and 250 μL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

[00233] 9.3 – Enzyme linked immuno-sorbent assay (ELISA).

[00234] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-12 antibody (0.5 μg/mL) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 μL standard AAD-12 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve, purified AAD-12 was diluted 2-fold serially from 250 to 3.9 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-12 antibody-HRP conjugate (0.5 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce, Rockford, IL), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 μL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-12 in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[00235] For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

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[00236] 9.4 – Western blotting analysis.

[00237] Plant extracts or AAD-12 standards (5 and 0.5 µg/mL) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-12 protein was detected by anti-AAD-12 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham, NJ).

Example 10 – Tobacco Transformation

[00238] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB3278, aka pDAS1580, *AAD-12 (v1) + PAT*), grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30°C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma).

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These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

[00239] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[00240] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-12 (v1)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00241] The DNA samples were diluted to 9 ng/µl and then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

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Table 13 Tobacco T0 events transformed with pDAS1580 (*AAD-12 (v1) + PAT*)

# Tube	Plant ID	Copy # PAT	PTU PCR AAD12	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance*
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+	*	*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable
24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High

* Discount of relative resistance made by tolerance to 560 g ae/ha fluroxypyr.

[00242] All events were also assayed for the presence of the *AAD-12 (v1)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR *AAD-12* were (SdpacodF ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:10) and (SdpacodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:11). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by

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72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably *AAD-12 (v1)* since these genes are physically linked) were regenerated and moved to the greenhouse.

[00243] 10.1 Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T_0 tobacco

[00244] T_0 plants from each of the 18 events were challenged with a wide range of 2,4-D, triclopyr, or fluroxypyr sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluroxypyr was likewise applied at 35, 140, or 560 g ae/ha. Triclopyr was appied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ae/ha (equivalent to 4X field rate). On the whole, *AAD-12 (v1)* events were more sensitive to fluroxypyr, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T0 plant responses to 560 g ae/ha fluroxypyr. Events were categorized into "low" (>40% injury 14 DAT), "medium" (20-40% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (**Table 13**).

[00245] 10.2 Verification of high 2,4-D tolerance in T_1 tobacco..

[00246] Two to four T_0 individuals surviving high rates of 2,4-D and fluroxypyr were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 7.4), followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (gene selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of 140 - 2240 g ae/ha 2,4-D dimethylamine salt (DMA), 70-1120 g

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ae/ha triclopyr or 35-560 g ae/ha fluroxypyr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity.

[00247] No injury was observed at 4X field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line but the greatest injury was observed with fluroxypyr, although this was short-lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT. (Table 14) It is important to note that untransformed tobacco is exceedingly sensitive to fluroxypyr. These results indicated commercial level 2,4-D tolerance can be provided by *AAD-12 (v1)*, even in a very auxin-sensitive dicot crop like tobacco and now across multiple species. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluroxypyr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

Table 14. Segregating *AAD-12* T_1 tobacco plants' response to phenoxy and pyridyloxy auxin herbicides.

Herbicide	KY160 – Wildtype	1580(1)-004 (high tolerance in T_0 generation)	1580(1)-018 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
140 g ae/ha 2,4-D DMA	45	0	0
560 g ae/ha 2,4-D DMA	60	0	0
2240 g ae/ha 2,4-D DMA	73	0	0
70 g ae/ha triclopyr	40	0	5
280 g ae/ha triclopyr	65	0	5
1120 g ae/ha triclopyr	80	0	8
35 g ae/ha fluroxypyr	85	0	8
140 g ae/ha fluroxypyr	93	0	10
560 g ae/ha fluroxypyr	100	3	18

[00248] 10.3 *AAD-12 (v1)* heritability in tobacco

[00249] A 100 plant progeny test was also conducted on seven T1 lines of *AAD-12 (v1)* lines. The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants were then sprayed with 560 g

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ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

10.4 – Field tolerance of pDAS1580 tobacco plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

[00250] Field level tolerance trials were conducted on three *AAD-12 (v1)* lines (events PDAS1580-[1]-018.001, PDAS1580-[1]-004.001 and PDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Mississippi. Tobacco transplants were grown in the greenhouse by planting T1 seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

[00251] The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after application.

[00252] *AAD-12 (v1)* event response to 2,4-D, triclopyr, and fluroxypyr are shown in **Table 15**. The non-transformed tobacco line was severely injured (63% at 14DAT) by 2,4-D at 560 g ae/ha which is considered the 1X rate. The *AAD-12 (v1)* lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8X rates, respectively. The non-transformed tobacco line was severely injured (53% at 14DAT) by the 2X rates of triclopyr (840 g ae/ha). *AAD-12 (v1)* lines demonstrated tolerance 2X rates of triclopyr with an average of 5% injury at 14DAT across the three lines. Fluroxypyr at 280 g ae/ha caused severe injury (99%) to the non-transformed line at 14DAA. *AAD-12 (v1)* lines demonstrated increased tolerance with an average of 11% injury at 14DAT.

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[00253] These results indicate that *AAD-12 (v1)* transformed event lines displayed a high level of resistance to 2,4-D, triclopyr and fluroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed tobacco.

Table 15. AAD-12 tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.

Herbicide Treatment		Average % Injury across locations at 14 DAT			
Active Ingredient	Rate	Wild type	PDAS1580-[1]-004.001	PDAS1580-[1]-020.016	PDAS1580-[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxypyr	280 GM AE/HA	99	11	11	12

[00254] 10.5 Stacking of *AAD-12* to increase herbicide spectrum

[00255] *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) plants (*see* PCT/US2005/014737 for the latter) were reciprocally crossed and F_1 seed was collected. F_1 seed will be stratified and treated with 560 g ai/ha glufosinate. To the surviving plants, 560 g ae/ha dichlorprop (to confirm the presence of the *AAD-1 (v3)* gene) + 560 g ae/ha triclopyr dichlorprop (to confirm the presence of the *AAD-12 (v1)* gene) will be applied to the plants under the same spray regimen as used for the other testing. Plants shall be graded at 3 and 14 DAT. Tissue samples are taken from the F_1 plants and subjected to Western analysis to confirm the presence of both genes. The results will confirm that *AAD-12 (v1)* can be successfully stacked with *AAD-1 (v3)*, thus increasing the spectrum herbicides that may be applied to the crop of interest (phenoxyactetic acids + phenoxypropionic acids vs penoxyacetic acids + pyridyloxyacetic acids for AAD-1 and AAD-12, respectively). The complementary nature of herbicide cross resistance patterns allows convenient use of these two genes as conventionally stackable selectable markers.

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Example 11 - Soybean Transformation

[00256] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00257] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-12 (v1)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-12 (v1)*.

[00258] 11.1 Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00259] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly

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dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00260] 11.1.1 *Agrobacterium* preparation. The plasmid pDAB3759 contains the *AAD-12 (v1)* gene under the control of the *Casava vein mosaic virus* promoter. This plasmid also carries the *PAT* gene under the control of *Arabidopsis* Ubi10 promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. PDAB3759 also carries the glyphosate resistance trait described in section 2.4. This vector can be used in the transformation experiment described below. The construct pDAB3759 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

[00261] *Agrobacterium* cultures harboring pDAB3759 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

[00262] 11.1.2 – Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be

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rinsed five times with sterile water on hormone-free SHGA medium and allowed to germinate for 5 days at 24° C, with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.*, 1988).

[00263] Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 μM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

[00264] Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin , 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

[00265] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D

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selection pressure ranging from10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

[00266] The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

[00267] 11.1.3 – Progeny evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-12 (v1)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance will be assessed in the T_1 generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of *AAD-12 (v1)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

[00268] 11.2 Transformation Method 2: Aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00269] Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Patent No. 6,809,232 (Held *et al.*).

[00270] Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144, 96E692, or 180801500-17 were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3277 (see Table 7) using linearized DNA at a concentration of approximately 0.2 µg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos.

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With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing 1/2 B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

[00271] Variations of media referred to in **Table 16** were tested, *e.g.*, B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and 1/2 B5G which contained all ingredients of B5G medium at half strength.

[00272] Plants were selected after 5-8 months in selection and molecular analysis was carried out to check the integration of the transgene. Two T_0 events were generated and were positive by PCR for the presence of the AAD-12 gene. One plant was fertile and produced 12 normal T_1 seeds that will be tested for 2,4-D resistance as described in section 11.1.

Table 16. Growth Media for Soybean

	Ingredients in 1 Liter		
	B1-30	**B3**	**B5G**
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8g	8g	
Gelrite			2g
pH	5.8	5.8	5.8

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Example 12– *AAD-12 (v1)* in Cotton

[00273] 12.1 – Cotton transformation protocol.

[00274] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (**Table 17**) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00275] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (**Table 17**) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (**Table 17**) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (**Table 17**). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00276] *Agrobacterium* preparation. Inoculate 35ml of Y media (**Table 17**) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (**Table 17**) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

[00277] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison, MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.05mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for to the AAD-12 enzyme. Callus is then transferred to CG-media (**Table 17**), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (**Table 17**) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media,

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embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

[00278] Larger, well-developed embryos are isolated and transferred to DK media (**Table 17**) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

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Table 17. Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

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[00279] 12.2 – Experiment specifics.

[00280] For this experiment 500 cotyledon segments were treated with pDAB724. The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage; then callus lines that are embryogenic will be sent for Western analysis.

[00281] 12.3 – Plant regeneration.

[00282] *AAD-12 (v1)* cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the *AAD-12 (v1)* gene provides resistance to 2,4-D in cotton, both the *AAD-12 (v1)* cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187 L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T_1 seed or outcrossed with an elite cotton line to produce F_1 seed. The subsequent seed produced will be planted and evaluated for herbicide resistance as previously described.

Example 13 – *Agrobacterium* Transformation of Other Crops

[00283] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g.,* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.*, 1997.

[00284] The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *AAD-12 (v1)*, for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato

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(*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa*, *perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans (*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, and other families), and Clover (*Leguminosae*). Such plants, with *AAD-12 (v1)* genes, for example, are included in the subject invention.

[00285] *AAD-12 (v1)* has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluroxypyr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited to: alder, ash, aspen, beech, birch, cherry, eucalyptys, hickory, maple, oak, pine, and poplar. Use of auxin resistance for the selective control in ornamental species is also within the scope of this invention. Examples could include, but not be limited to, roses, Euonymus, petunia, begonia, and marigolds.

Example 14 – Further Evidence of Surprising Results: *AAD-12 vs. AAD-2*

[00286] 14.1 – *AAD-2 (v1)* initial cloning.

[00287] Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

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[00288] The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

[00289] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H_2O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00290] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO:6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:7) primers, per manufacturers

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instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[00291] 14.2 – Completion of *AAD-2 (v1)* binary vector.

[00292] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. *See* PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:12) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:13) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI – NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

[00293] 14.3 – Evaluation of transformed *Arabidopsis*.

[00294] Freshly harvested T_1 seed transformed with a plant optimized *AAD-12 (v1)* or native *AAD-2 (v1)* gene were planted and selected for resistance to glufosinate as previously described Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

[00295] *AAD-12 (v1)* and *AAD-2 (v1)* did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants.

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Surprisingly, *AAD-2 (v1)*and *AAD-2 (v2)* transformants were far less resistant to 2,4-D than *AAD-12 (v1)* genes.both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (*see* PCT/US2005/014737). Conversely, AAD-12 had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D (Table 10). Tolerance improved slightly for plant-optimized *AAD2 (v2)* versus the native gene; however, comparison of both *AAD-12* and *-2* plant optimized genes indicates a significant advantage for *AAD-12 (v1) in planta.*

[00296] These results are unexpected given that *in vitro* comparison native microbial genes of *AAD-2 (v1)* (*see* PCT/US2005/014737) and *AAD-12 (v2)* indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. No large difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes (*see* PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of AAD-12 (v1) *in planta* for imparting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides is unexpected.

Example 15 – Preplant Burndown Applications

[00297] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

[00298] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and

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activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (*see* federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With *AAD-12 (v1)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. Likewise, the unique attributes of AAD-12 allowing degradation of triclopyr and fluroxypyr increase utility by allowing substitution or tank mixesof 70-1120 or 35-560 g ae/ha of triclopyr andfluroxypyr, respectively, to increase spectram and/or increase the abilty to control perennial or viney weed species.

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Example 16 – In-Crop Use of Phenoxy Auxins Herbicides in Soybeans, Cotton, and Other Dicot Crops Transformed Only with *AAD-12 (v1)*

[00299] *AAD-12 (v1)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D and MCPA) and pyridyloxy auxins (trickopyr and fluroxypyr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00300] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluroxypyr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

[00301] Further benefits could include tolerance to 2,4-D, triclopyr or fluroxypyr required before planting following aryloxyacetic acid auxin herbicide application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D, triclopyr or fluroxypyr. Dicamba (and other herbicides) can still be used for the subsequent control of *AAD-12 (v1)*-transformed dicot crop volunteers.

[00302] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the *AAD-12 (v1)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by *AAD-12 (v1)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

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Example 17 - In-Crop Use of Phenoxy Auxin and Pyridyl Auxin Herbicides in *AAD-12 (v1)* Only Transformed Corn, Rice, and Other Monocot Species

[00303] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-12 (v1)* would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D,.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. *AAD-12 (v1)*-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 – 1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

[00304] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, or fluroxypyr. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-12 (v1)* HTC combination strategy, whether one rotates crops species or not. The increased tolerance of corn, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 18 – *AAD-12 (v1)* Stacked With Glyphosate Tolerance Trait in Any Crop

[00305] The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional

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GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-12 (v1)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-12 (v1)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more

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preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Tricopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimin. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D, triclopyr, or fluroxypyr.

[00306] One skilled in the art of weed control will recognize that use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 19 - *AAD-12 (v1)* Stacked with Glufosinate Tolerance Trait in Any Crop

[00307] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

[00308] By stacking *AAD-12 (v1)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy,

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flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

   i) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species. Triclopyr and fluroxypyr would be acceptable components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) A multiple combination of glufosinate (200-500 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00309] One skilled in the art of weed control will recognize that use of one or more commercial aryloxyacetic auxin herbicides alone or in combination (sequentially or independently) is enabled by *AAD-12 (v1)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each

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alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - *AAD-12 (v1)* Stacked with *AHAS* Trait in Any Crop

[00310] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* Commercial imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking *AAD-12 (v1)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-12 (v1)*, monoct crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and these auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-12 (v1)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. For triclopyr, application rates would

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typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp.* can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. See rates above for triclopyr or fluroxypyr. iii)

b) A mulitple combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) +/- 2,4-D (280-1120 g ae/ha) +/- triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00311] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by *AAD-12 (v1)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-12 (v1)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-12 (v1)* in Rice

[00312] 21.1 – Media description.

[00313] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

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[00314] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3–4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (1/2MSH50).

[00315] 21.2 – Tissue culture development.

[00316] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants

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regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week prior to storage at 4° C.

[00317] 21.3 – Microprojectile bombardment.

[00318] All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB4101, 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[00319] 21.4 – Tolerance testing.

[00320] Rice plantlets at the 3-5 leaf stage will be sprayed with a selective dose (~70-140 g ae/ha) of imazethapyr + methylated seed oil (1%) by track sprayer at 187 L/ha spray volume. Rating for sensitivity or resistance will be done at 10-14 days after treatment (DAT).

[00321] 21.5 – Tissue harvesting, DNA isolation and quantification.

[00322] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

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Example 22 – *AAD-12 (v1)* in Canola

[00323] 22.1 – Canola transformation.

[00324] The *AAD-12 (v1)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained *AAD-12 (v1)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi10 promoter (*see* section 2.4).

[00325] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00326] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00327] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00328] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 2-3 weeks.

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[00329] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/l MES, 5 or 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00330] The transformed T_0 plants will be self-pollinated in the greenhouse to obtain T_1 seed. The T_0 plants and T_1 progeny will be sprayed with a range of herbicide (2,4-D; triclopyr; fluroxypyr; glyphosate; and glufosinate) rates to establish the level of protection by the *AAD-12 (v1)* gene as previously described above.

[00331] 22.2 – "Molecular analysis": canola materials and methods

[00332] 22.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ul.

[00333] 22.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-12 (v1)* were (SEQ ID NO:10) (forward) and (SEQ ID NO:11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-12 (v1)* events tested positive. Three negative control samples tested negative.

[00334] 22.2.3 – ELISA.

[00335] Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have

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minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 18.

Table 18 Expression of AAD-12 in Canola plants

Sample #	[TSP] (µg/mL)	[AAD-12] (ng/mL)	Expression (ppm TSP) (ELISA)	Western
31	5614.96	1692.12	301.36	++++
33	4988.26	2121.52	425.30	++++
38	5372.25	3879.09	722.06	++++
39	2812.77	41.36	14.71	+
40	3691.48	468.74	126.98	+++
Control 1	2736.24	0.00	0.00	-
Control 2	2176.06	0.00	0.00	-
Control 3	3403.26	0.00	0.00	-

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Claims

1. A transgenic plant comprising a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide.

2. The transgenic plant of claim 1, said polynucleotide also conferring resistance to a phenoxy auxin herbicide.

3. A method of controlling at least one weed in a field, said method comprising growing at least one transgenic plant of claim 1 in said field, and applying a pyridyloxy auxin herbicide to at least a portion of said field.

4. A method of controlling at least one weed in a field, said method comprising growing at least one plant of claim 2 in said field, and applying a phenoxy auxin herbicide to at least a portion of said field.

5. The method of claim 3, further comprising applying a phenoxy auxin herbicide to at least a portion of said field.

6. The method of claim 3, said pyridyloxy auxin herbicide selected from the group consisting of fluroxypyr and triclopyr.

7. The method of claim 4, said phenoxy auxin herbicide selected from the group consisting of 2,4-D and MCPA.

8. The plant of claim 1, said plant further comprising at least one additional herbicide resistance gene.

9. The plant of claim 8, said at least one additional herbicide resistance gene rendering said plant resistant to at least one herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

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10. The plant of claim 1 wherein said plant is selected from the group consisting of soybean, canola, tobacco, Arabidopsis, corn, cotton, and rice.

11. The plant of claim 1 wherein said polynucleotide encodes an AAD-12 protein having aryloxyalkanoate dioxygenase activity, said AAD-12 protein having at least 95% identity to a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

12. The plant of claim 1 wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

13. A polynucleotide comprising a codon composition that is optimized for expression in an organism other than *Delftia acidovoransa*, wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity.

14. The polynucleotide of claim 13 wherein said polynucleotide comprises SEQ ID NO:5.

15. The polynucleotide of claim 13 wherein said codon composition that is optimized for expression in a plant.

16. The polynucleotide of claim 15 wherein said polynucleotide comprises SEQ ID NO:3.

17. A plant cell comprising a heterologous polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

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18. A method of selecting for a transformed plant cell of claim 17 wherein said method comprises subjecting a plurality of plant cells to transformation with said polynucleotide, then growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin herbicide.

19. The method of claim 18 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

20. A seed of a plant of claim 1.

21. A plant grown from the seed of claim 20.

22. A part, progeny, or asexual propagate of the plant of claim 1.

23. The method of claim 4 wherein said method is used to treat and/or prevent herbicide-resistant weeds, and/or to treat weeds that are naturally tolerant to control by other herbicides.

24. The method of claim 5 wherein said method further comprises applying a third herbicide.

25. The method of claim 24 wherein said phenoxy auxin herbicide is 2,4-D; said pyridyloxy auxin herbicide is selected from the group consisting of triclopyr and fluroxypyr; and said third herbicide is selected from the group consisting of glyphosate, glufosinate, paraquat, chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron, cloransulam-methyl, diclosulam, dicamba, florasulam, flumetsulam, metosulam, penoxsulam imazamox, imazethapyr, imazaquin, dicamba, mesotrione, isoxaflutole, sulcotrione, flufenpyr, pyraflufen, butafenacil, amidosulfuron, bensulfuron, chlorimuron, cinosulfuron, flupyrsulfuron, foramsulfuron, primisulfuron, prosulfuron,

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rimsulfuron, thifensulfuron, triasulfuron, triflusulfuron, flumiclorac, flumioxazin, fluthiacet, carfentrazone, sulfentrazone, acifluorfen, fomesafen, lactofen, and oxyfluorfen.

26. A method of controlling at least one weed in a field, said method comprising planting in said field seed of at least one transgenic plant, said plant comprising

a heterologous polynucleotide that encodes an enzyme conferring resistance to a pyridyloxy auxin herbicide, and

a second heterologous polynucleotide encodes an enzyme conferring resistance to at least one other herbicide;

said method further comprising applying to at least a portion of said field a first herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide; and applying said at least one other herbicide to said at least a portion of said field.

27. The method of claim 26 wherein said herbicides are applied sequentially or concurrently.

28. The method of claim 26 wherein said first herbicide is a phenoxy auxin herbicide.

29. The method of claim 26 wherein said first herbicide is selected from the group consisting of 2,4-D, triclopyr, and fluroxypyr.

30. The method of claim 26 wherein said at least one other herbicide is selected from the group consisting of glyphosate, glufosinate, paraquat, chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron, cloransulam-methyl, diclosulam, dicamba florasulam, flumetsulam, metosulam, penoxsulam imazamox, imazethapyr, imazaquin, dicamba, mesotrione, isoxaflutole, sulcotrione, flufenpyr, pyraflufen, butafenacil, amidosulfuron, bensulfuron, chlorimuron, cinosulfuron, flupyrsulfuron, foramsulfuron, primisulfuron, prosulfuron, rimsulfuron, thifensulfuron, triasulfuron, triflusulfuron, flumiclorac, flumioxazin, fluthiacet, carfentrazone, sulfentrazone, acifluorfen, fomesafen, lactofen, and oxyfluorfen.

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31. A method of assaying a protein for its ability to cleave a pyridyloxyalkanoate herbicide, said method comprising providing a sample comprising said protein and a pyridyloxy auxin herbicide, and assaying said sample for presence of a chloropyridinol by ultraviolet absorption.

32. A method of assaying a protein for aryloxyalkanoate dioxygenase activity wherein said method comprises providing a sample comprising said protein and 2-(2-chloro,4-nitrophenoxy) propionate and/or acetate, and colorimetrically assaying said sample for presence of a 2-chloro,4-nitrophenol.

33. An expression vector comprising a nucleic acid molecule encoding a protein that enzymatically degrades at least one herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein said nucleic acid molecule hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

34. The expression vector of claim 33 wherein said promoter is a plant promoter.

35. The expression vector of claim 33 comprising a promoter selected from a cassava vein mosaic virus promoter, CaMV 35S promoter, Figwort Mosaic Virus promoter, rice actin promoter, phaseolin promoter, *Arabidopsis thaliana* Ubiquitin 10 promoter, maize ubiquitin promoter, *Arabidopsis thaliana* Act2 promoter, *Arabidopsis thaliana* Ubiquitin 11 promoter, and *Arabidopsis thaliana* Ubiquitin 3 promoter.

36. The method of claim 4 wherein said plant is resistant to a herbicide formulation comprising glyphosate.

37. A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said first herbicide is selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin.

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38. The method of claim 37 wherein said first herbicide is an acid, an inorganic salt, an organic salt, or an ester.

39. The method of claim 37 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

40. The method of claim 39 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate.

41. A method of detecting whether a plant comprises a polynucleotide of claim 15 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

42. The method of claim 41 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

43. The method of 41 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

44. The method of 42 wherein said method comprises using an antibody to detect for the presence of said protein.

45. The plant of claim 1 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

46. The plant of claim 1 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

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47. The method of claim 4 wherein said plant is resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, imazethapyr, chlorsulfuron, dicamba, mesotrione, isoxaflutole, and butafenacil.

48. The method of claim 4 wherein said herbicide is an achiral phenoxy auxin.

49. The method of claim 26 wherein said plant is a monocot.

50. The method of claim 49 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, warm and cool-season turf grass, oats, sorghum, and pasture grasses.

51. The method of claim 26 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

52. The method of claim 51 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

53. A method of conferring herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a polynucleotide encoding an enzyme capable of using a pyridyloxy auxin herbicide and a phenoxy auxin herbicide as a substrate.

54. A method of controlling glyphosate-resistant, naturally glyphosate tolerant, and/or naturally glufosinate tolerant weeds in a field of glyphosate and/or glufosinate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 13, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

55. The method of claim 54 wherein said herbicide is a phenoxy auxin.

56. The method of claim 55 wherein said phenoxy auxin is 2,4-D.

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57. A method of controlling weeds that are resistant to an ALS-inhibiting herbicide in a field of crop plants that comprise a polynucleotide of claim 13, said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

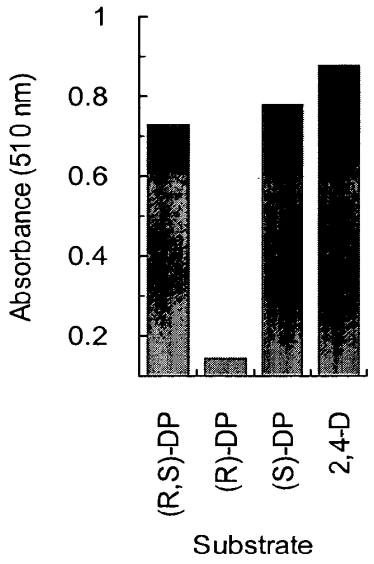
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Abstract

[00336] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-12* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide tolerant crop trait and selectable marker opportunities.

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Figure 1. Activity of AAD-12(v2) on 2,4-D and enantiomers of dichlorprop. 4.4 µg AAD-12(v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of α-ketoglutarate. After 5 min the reaction was quenched and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.



J:\DAS\DAS-129P\Figure 1.doc

Sequences

SEQ ID NO:1
Native *aad12* coding region
879 bases

```
ATGCAGACGA CGCTGCAGAT TACCCCCACA GGCGCCACCC TGGGCGCCAC CGTCACCGGC
GTGCACCTGG CCACGCTGGA CGACGCCGGC TTCGCCGCCC TGCACGCCGC CTGGCTGCAG
CATGCGCTGC TGATCTTCCC CGGCCAGCAC CTCAGCAACG ACCAGCAGAT CACTTTTGCC
AAACGCTTCG GCGCGATCGA GCGCATCGGC GGCGGCGACA TCGTGGCCAT CTCCAATGTC
AAGGCCGATG GCACGGTGCG CCAGCACAGC CCCGCCGAGT GGGACGACAT GATGAAGGTC
ATCGTCGGCA ACATGGCCTG GCATGCCGAC AGCACCTACA TGCCGGTGAT GGCGCAGGGC
GCGGTGTTCT CGGCCGAAGT GGTGCCCGCA GTGGGCGGGC GCACCTGCTT CGCCGACATG
CGCGCCGCCT ACGACGCGCT GGACGAGGCC ACCCGCGCCC TGGTGCACCA GCGCTCGGCG
CGGCATTCGC TGGTGTATTC GCAGAGCAAG CTGGGCCATG TGCAGCAGGC CGGCTCGGCC
TACATCGGCT ACGGCATGGA CACCACCGCC ACGCCCCTGC GCCCGCTGGT CAAGGTGCAT
CCCGAGACCG GCCGCCCCTC GCTGCTGATC GGCCGCCATG CCCATGCCAT CCCGGGCATG
GACGCCGCCG AATCCGAGCG CTTCCTGGAA GGCCTGGTCG ACTGGGCCTG CCAGGCGCCG
CGGGTGCATG CCCACCAATG GGCCGCCGGC GACGTGGTGG TGTGGGACAA CCGCTGCCTG
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SEQ ID NO:2
Aad12 protein encoded by SEQ ID NO:1
292 amino acids

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Phe Pro Gly Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala
Lys Arg Phe Gly Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val
Ala Ile Ser Asn Val Lys Ala Asp Gly Thr Val Arg Gln His Ser
Pro Ala Glu Trp Asp Asp Met Met Lys Val Ile Val Gly Asn Met
Ala Trp His Ala Asp Ser Thr Tyr Met Pro Val Met Ala Gln Gly
Ala Val Phe Ser Ala Glu Val Val Pro Ala Val Gly Gly Arg Thr
Cys Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Glu Ala
Thr Arg Ala Leu Val His Gln Arg Ser Ala Arg His Ser Leu Val
Tyr Ser Gln Ser Lys Leu Gly His Val Gln Gln Ala Gly Ser Ala
Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr Pro Leu Arg Pro
Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser Leu Leu Ile
Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala Glu Ser
Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala Pro
Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp
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Thr Glu Gly Ala Ala Leu Val
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SEQ ID NO:3
Plant-optimized DNA sequence (*aad12* v2)
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GCTAAACGCT TTGGAGCAAT TGAGAGGATT GGCGGAGGTG ACATTGTTGC CATATCCAAT
```

GTCAAGGCAG ATGGCACAGT GCGCCAGCAC TCTCCTGCTG AGTGGGATGA CATGATGAAG
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CTCGCTGGAC GCCCAGAAAC TGAGGGTGCT GCCTTGGTTT GA

SEQ ID NO:4
Aad12 protein encoded by SEQ ID NO:3
293 amino acids

Met Ala Gln Thr Thr Leu Gln Ile Thr Pro Thr Gly Ala Thr Leu
Gly Ala Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala
Gly Phe Ala Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu
Ile Phe Pro Gly Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe
Ala Lys Arg Phe Gly Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile
Val Ala Ile Ser Asn Val Lys Ala Asp Gly Thr Val Arg Gln His
Ser Pro Ala Glu Trp Asp Asp Met Met Lys Val Ile Val Gly Asn
Met Ala Trp His Ala Asp Ser Thr Tyr Met Pro Val Met Ala Gln
Gly Ala Val Phe Ser Ala Glu Val Val Pro Ala Val Gly Gly Arg
Thr Cys Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Glu
Ala Thr Arg Ala Leu Val His Gln Arg Ser Ala Arg His Ser Leu
Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln Gln Ala Gly Ser
Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr Pro Leu Arg
Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser Leu Leu
Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala Glu
Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala
Pro Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val
Trp Asp Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe
Lys Leu Pro Arg Val Met Trp His Ser Arg Leu Ala Gly Arg Pro
Glu Thr Glu Gly Ala Ala Leu Val

SEQ ID NO:5
E. coli-optimized DNA sequence (v3)
882 bases

ATGGCTCAGA CTACCCTGCA GATTACCCCG ACTGGTGCGA CCCTGGGTGC AACCGTTACC
GGCGTTCACC TGGCGACTCT GGATGACGCA GGTTTCGCTG CGCTGCACGC GGCTTGGCTG
CAACATGCTC TCCTGATTTT CCCAGGTCAG CACCTGTCCA ACGACCAGCA AATCACTTTT
GCAAAACGCT TCGGTGCGAT CGAACGTATC GGTGGCGGTG ATATTGTGGC GATCTCCAAC
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CTGGCAGGCC GCCCAGAAAC CGAGGGCGCG GCTCTGGTTT AA

SEQ ID NO:6
M13 forward sequencing primer
GTAAAACGAC GGCCAG

SEQ ID NO:7
M13 reverse sequencing primer
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SEQ ID NO:8
Forward PTU primer
ATAATGCCAG CCTGTTAAAC GCC

SEQ ID NO:9
Reverse PTU primer
CTCAAGCATA TGAATGACCT CGA

SEQ ID NO:10
SdpacodF: *AAD-12 (v1)* forward coding region primer
ATGGCTCATG CTGCCCTCAG CC

SEQ ID NO:11
SdpacodR: *AAD-12 (v1)* reverse coding region primer
CGGGCAGGCC TAACTCCACC AA

SEQ ID NO:12
primer "NcoI of Brady"
TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT

SEQ ID NO:13
primer "SacI of Brady"
GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC

I hereby certify that this correspondence is being electronically filed in the United States Patent and Trademark Office on Nov. 3, 2008

Jay M. Sanders, Patent Attorney

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors	Terry R. Wright et al	)	Examiner: Not Yet Assigned
Invention:	NOVEL HERBICIDE RESISTANCE GENES	)	Group No. Not Yet Assigned
Serial No.:	12/091,896	)	Confirmation No. 6482
Filing Date:	April 28, 2008	)	
Attorney Docket No.:	DAS-P0129-02-US (formerly DAS-129XC1)	)	

RESPONSE TO THE NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Dear Sir/Madam:

In response to the Notification of Missing Requirements Under 35 U.S.C. mailed on August 4, 2008, (a copy of which is attached), Applicants submit herewith a fully executed Declaration for Patent Application English Language Declaration by inventors Terry R. Wright, Justin M. Lira, Terence A. Walsh, Donald J. Merlo, Jayakumar P. Samuel and Gaofeng Ling.

Applicants also submit herewith a copy of the International Search Report in response to the additional defects noted by the United States Patent and Trademark Office.

The Commissioner is hereby authorized to charge to Baker & Daniels LLP's Deposit Account No. 02-0390 the amount of $130.00, which covers the fee for the late filing of the Declaration and a one-month extension fee of $120.00. The Commissioner is hereby authorized to charge any additional fees that may be due in connection with this Response to Baker & Daniels LLP's Deposit Account No. 02-0390.

Respectfully submitted,

BAKER & DANIELS LLP

Jay M. Sanders
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Date Submitted: November 3, 2008
Attachments

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
12/091,896	Terry R. Wright	DAS-129XC1

23557
SALIWANCHIK LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
PO BOX 142950
GAINESVILLE, FL 32614-2950

JMS RECEIVED RLR
AUG - 7 2008

INTERNATIONAL APPLICATION NO.	
PCT/US2006/042133	
I.A. FILING DATE	PRIORITY DATE
10/27/2006	10/28/2005

CONFIRMATION NO. 6428
371 FORMALITIES LETTER

OC000000031355282

Date Mailed: 08/04/2008

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as a Designated Office (37 CFR 1.494):

- Priority Document
- Copy of the International Application filed on 04/28/2008
- Biochemical Sequence Diskette filed on 04/28/2008
- Biochemical Sequence Listing filed on 04/28/2008
- U.S. Basic National Fees filed on 04/28/2008
- Priority Documents filed on 04/28/2008
- Specification filed on 04/28/2008
- Claims filed on 04/28/2008
- Abstracts filed on 04/28/2008
- Drawings filed on 04/28/2008
- Paper nucleotide sequence listings filed on 04/28/2008

The applicant needs to satisfy supplemental fees problems indicated below.

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.
- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.492(h) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

SUMMARY OF FEES DUE:

Total additional fees required for this application is **$130** for a Large Entity:

- **$130** Surcharge.

Additionally the following defects have been observed:

- The International Search Report has not be supplied, the Search Fee has been adjusted to reflect this omission.

FORM PCT/DO/EO/905 (371 Formalities Notice)

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 32 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

CHARITTA A SHELTON

Telephone: (703) 308-9140 EXT 207

FORM PCT/DO/EO/905 (371 Formalities Notice)

Declaration for Patent Application English Language Declaration	Attorney Docket No.	63979A
	First Named Inventor	Terry R. Wright
	COMPLETE IF KNOWN:	
☐ Submitted with initial filing ☒ Submitted after initial filing (surcharge required 37 CFR 1.16(e))	Application No.	12/091,896
	Filing Date	October 27, 2006
	Art Unit	
	Examiner	

As a below named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NOVEL HERBICIDE RESISTANCE GENES

the specification of which

☐ is attached hereto

OR

☒ was filed on 10/27/2006

as United States Application No. or PCT International Application No. 12/091,896

and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign applications(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's right certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

(Number)	(Country)	(Filing Date)	Priority Not Claimed	Certified Copy Attached YES	Certified Copy Attached NO
PCT/US2006/042133	PCT	October 27, 2006	☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional prior foreign applications are listed on a supplemental data sheet attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Terry R. Wright	
Sole or first inventor's signature	Date 03/13/07
Residence Westfield, Indiana	
Citizenship US	
Mailing Address 14162 Charity Chase Circle Westfield, Indiana 46074 UNITED STATES OF AMERICA	

Full name of second inventor Justin M. Lira	
Second inventor's signature	Date 03-12-07
Residence Fishers, Indiana	
Citizenship US	
Mailing Address 12373 Ensley Drive Fishers, Indiana 46038 UNITED STATES OF AMERICA	

Full name of third inventor Terence A. Walsh	
Third inventor's signature	Date 03/12/2007
Residence Zionsville, Indiana	
Citizenship US	
Mailing Address 11638 Promontory Trail Zionsville, Indiana 46077 UNITED STATES OF AMERICA	

Corteva Exhibit 2014 - Page 643 of 1122
Inari v. Corteva - PGR2023-00022

Full name of fourth inventor, if any	
Donald J. Merlo	
Fourth inventor's signature Donald J Merlo	Date March 12, 2007
Residence Carmel, IN	
Citizenship US	
Mailing Address 11845 Durbin Drive Carmel, Indiana 46032 UNITED STATES OF AMERICA	

Full name of fifth inventor, if any	
Jayakumar P. Samuel	
Fifth inventor's signature	Date 3.12.07
Residence Carmel, Indiana	
Citizenship US	
Mailing Address 3336 Kilkenny Circle Carmel, Indiana 46032 UNITED STATES OF AMERICA	

Full name of sixth inventor, if any	
Gaofeng Lin	
Sixth inventor's signature	Date 3/12/07
Residence Zionsville, Indiana	
Citizenship US	
Mailing Address 981 Franklin Trace Zionsville, Indiana 46077 UNITED STATES OF AMERICA	

Corteva Exhibit 2014 - Page 644 of 1122
Inari v. Corteva - PGR2023-00022

PATENT COOPERATION TREATY

RECEIVED
MAR 3 1 2008

From the INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing (*day/month/year*)
Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US06/42133	International filing date (*day/month/year*) 27 October 2006 (27.10.2006)
Applicant DOW AGROSCIENCES LLC	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70.

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 *bis*.1 and 90 *bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/ US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized Officer Vinod Kumar Telephone No. (571) 272-4445

Form PCT/ISA/220 (April 2002) *(See notes on accompanying sheet)*

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing (*day/month/year*) 28 MAR 2008
Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US06/42133	International filing date (*day/month/year*) 27 October 2006 (27.10.2006)
Applicant DOW AGROSCIENCES LLC	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70.

   For more detailed instructions, see the notes on the accompanying sheet.

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3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 *bis*.1 and 90 *bis*.3, respectively, before the completion of the technical preparations for international publication.

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/ US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Vinod Kumar Telephone No. (571) 272-4445

Form PCT/ISA/220 (April 2002) *(See notes on accompanying sheet)*

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US06/42133	International filing date (*day/month/year*) 27 October 2006 (27.10.2006)	(Earliest) Priority Date (*day/month/year*) 28 October 2005 (28.10.2005)
Applicant DOW AGROSCIENCES LLC		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the Report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   ☒ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   ☒ contained in the international application in written form.
   ☒ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. ☐ **Certain claims were found unsearchable** (See Box I).
3. ☒ **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____
   ☐ as suggested by the applicant.
   ☐ because the applicant failed to suggest a figure.
   ☐ because this figure better characterizes the invention.
   ☒ None of the figures

Form PCT/ISA/210 (first sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/42133

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-84 (SEQ ID NOs: 1 and 2)

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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Inari v. Corteva - PGR2023-00022

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US06/42133

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8): **A01H 5/00**(2006.01);**C12N 15/82**(2006.01);**C12N 5/04**(2006.01)

USPC: 800/295;800/300;800/301;800/302;800/278;800/289;435/468;435/419
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 800/295; 800/300; 8000/301; 8000/302; 8000/278; 8000/289; 435/468; 435/419

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Kaphammer et al. (US Patent No. 5,608,147, Issued March 4, 1997).	15-21, 30-32, 73-76
---		---
Y		42-43, 77
Y	Perlak et al. (PNAS, 88:3324-3328, 1991).	42
Y	Kasuga et al. (Nature Biotechnology, 17:287-291, 1999).	43
Y	Verdaguer et al. (Plant Molecular Biology, 31:1129-1139, 1996).	77

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
28 November 2007 (28.11.2007)

Date of mailing of the international search report
28 MAR 2008

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner of Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (571) 273-3201

Authorized officer
Vinod Kumar
Telephone No. (571) 272-4445

Form PCT/ISA/210 (second sheet) (July 1998)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-84 drawn to a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 1, expression cassette, transgenic plant cell, or transgenic plant comprising said polynucleotide.

Group II, claim(s) 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-84 drawn to a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 3, expression cassette, transgenic plant cell, or transgenic plant comprising said polynucleotide.

Group III, claim(s) 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81 and 83-84 drawn to a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 5, expression cassette, transgenic plant cell, or transgenic plant comprising said polynucleotide.

Group IV, claim(s) 5-7, 22-23, 66-67, and 78 drawn to a method of selecting for transgenic plant cell comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 1.

Group V, claim(s) 5-7, 22-23, 66-67, and 78 drawn to a method of selecting for transgenic plant cell comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 3.

Group VI, claim(s) 5-7, 22-23, 66-67, and 78 drawn to a method of selecting for transgenic plant cell comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 5.

Group VII, claim(s) 8-14, 24-27, 33-37, 44-59, 65, 68-72, 82, and 85-88 drawn to a method of controlling weeds or protecting crops comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 1.

Group VIII, claim(s) 8-14, 24-27, 33-37, 44-59, 65, 68-72, 82, and 85-88 drawn to a method of controlling weeds or protecting crops comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 3.

Group IX, claim(s) 8-14, 24-27, 33-37, 44-59, 65, 68-72, 82, and 85-88 drawn to a method of controlling weeds or protecting crops comprising expression of a polynucleotide encoding a polypeptide having aryloxyalkanote dioxygenase activity, wherein polynucleotide is defined in SEQ ID NO: 5.

Group X, claim(s) 28-29, 39, and 41 drawn to a method of assaying a protein, or wherein said protein is encoded by SEQ ID NO: 1.

Group XI, claim(s) 28-29, 39, and 41 drawn to a method of assaying a protein, or wherein said protein is encoded by SEQ ID NO: 3.

Group XII, claim(s) 28-29, 39, and 41 drawn to a method of assaying a protein, or wherein said protein is encoded by SEQ ID NO: 5.

Group XIII, claim(s) 38, and 40 drawn to a method of detecting a polynucleotide, or wherein said polynucleotide is defined in SEQ ID NO: 1.

Group XIV, claim(s) 38, and 40 drawn to a method of detecting a polynucleotide, or wherein said polynucleotide is defined in SEQ ID NO: 3.

Group XV, claim(s) 38, and 40 drawn to a method of detecting a polynucleotide, or wherein said polynucleotide is defined in SEQ ID NO: 5.

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INTERNATIONAL SEARCH REPORT

The inventions listed as Groups I-XV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking Groups I-XV appear to be a polynucleotide encoding a protein having aryloxyalkanoate dioxygenase activity. However, Schleinitz et al. (NCBI, GenBank Sequence Accession No. AY327575, Published September 8, 2004) teach a polynucleotide sequence encoding2, 4-dichlorophenoxypropionate cleavage. Therefore, the technical feature linking the inventions of Groups I-XV does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

Applicants are reminded that different nucleotide sequences and amino acid sequences are structurally distinct chemical compounds and are unrelated to one another. These sequences are thus deemed to normally constitute different inventive concepts.

Applicants are reminded that claim 27 is directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid.

Continuation of B. FIELDS SEARCHED Item 3:
WEST (USPT, PGPB), Agricola, CAplus, Biosis, Key word search: herbicide, aryloxyalkanoate dioxygenase, pyridyloxy auxin, 2, 4-D, bacillus thuringiensis, abiotic stress tolerance, insect resistance, phenoxy auxin, transgenic, transform, cassava vein mosaic virus promoter, fluroxypr, triclopyr.

Corteva Exhibit 2014 - Page 651 of 1122
Inari v. Corteva - PGR2023-00022

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	28 MAR 2008
Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US06/42133	27 October 2006 (27.10.2006)	28 October 2005 (28.10.2005)

International Patent Classification (IPC) or both national classification and IPC

IPC(8): **A01H 5/00**(2006.01);**C12N 15/82**(2006.01);**C12N 5/04**(2006.01)
USPC: 800/295;800/300;800/301;800/302;800/278;800/289;435/468;435/419

Applicant

DOW AGROSCIENCES LLC

1. This opinion contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☒	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis(b)* that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	01 December 2007 (01.12.2007)	Vinod Kumar Telephone No. (571) 272-4445

Form PCT/ISA/237 (cover sheet) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☒ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☒ on paper

☒ in electronic form

c. time of filing/furnishing

☒ contained in the international application as filed.

☒ filed together with the international application in electronic form.

☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:
   - ☐ paid additional fees
   - ☐ paid additional fees under protest and, where applicable, the protest fee
   - ☐ paid additional fees under protest but the applicable protest fee was not paid
   - ☒ not paid additional fees
2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
   - ☐ complied with
   - ☒ not complied with for the following reasons:

   See the lack of unity section of the International Search Report(Form PCT/ISA/210)

4. Consequently, this opinion has been established in respect of the following parts of the international application:
   - ☐ all parts.
   - ☒ the parts relating to claims Nos. 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-94 (SEQ ID NOs: 1 and 2)

Form PCT/ISA/237 (Box No. IV) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO
Inventive step (IS)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO
Industrial applicability (IA)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO

2. Citations and explanations:

Please See Continuation Sheet

Corteva Exhibit 2014 - Page 655 of 1122
Inari v. Corteva - PGR2023-00022

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 19-21, 30, 73, and 75 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims 19-21, 30, 73, and 75 not fully supported by the description. The application, as originally filed, did not describe genus of sequences that would hybridize to SEQ ID NO: 1. These claims encompass structures whose structures are not described in the specification, and thus one skilled in the art would not know their function. The stringent conditions described on page 22 of specification would encompass hybridization of unrelated nucleic acid sequences to instant SEQ ID NO: 1.

Claims 19-21, 30, 73, and 75 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because of the claims 19-21, 30, 73, and 75 are not fully supported by the description. The description does not disclose the claimed invention in a manner sufficiently clear and complete for the claimed invention to be carried out by a person skilled in the art because the stringent conditions described on page 22 of specification would encompass hybridization of unrelated nucleic acid sequences to instant SEQ ID NO: 1. One skilled in the art would not know how to use said unrelated sequences or plants having said unrelated sequences.

Corteva Exhibit 2014 - Page 656 of 1122
Inari v. Corteva - PGR2023-00022

Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

V.I. Reasoned Statements:
The opinion as to Novelty was positive (Yes)with respect to claims 1-4, 42-43, 60-64, 77, 79-81, 83-84
The opinion as to Novelty was negative (No) with respect to claims 15-21, 30-32, 73-76
The opinion as to Inventive Step was positive (Yes)with respect to claims 1-4, 60-64, 79-81, 83-84
The opinion as to Inventive Step was negative(NO) with respect to claims 15-21, 30-32, 42-43, 73-77
The opinion as to Industrial Applicability was positive (YES) with respect to claims 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, 83-84
The opinion as to Industrial Applicability was negative(NO) with respect to claims NONE

V. 2. Citations and Explanations:

Claims 1-4, 42-43, 60-64, 77, 79-81, and 83-84 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a transgenic plant cell plant or seed derived thereof, comprising a polynucleotide that encodes an AAD-12 protein of SEQ ID NO: 2, or a variant of SEQ ID NO: 2 having aryloxyalkanoate dioxygenase activity and exhibiting at least 95% sequence identity to SEQ ID NO: 2. The prior art does not teach or failry suggest that said plant further comprises a gene for insect or stress resistance. The prior art does not teach or suggest an isolated polynucleotide that encodes a protein that enzymatically degrades a phenoxy auxin or pyridyloxy auxin herbicide, and wherein said polynucleotide is operably linked with a cassava vein mosaic virus promoter.

Claims 15-21, 30-32, 73-76 lack novelty under PCT Article 33(2) as being anticipated by Kaphammer et al. (US Patent No. 5,608,147, Issued March 4, 1997).

Kaphammer et al. teach a transgenic plant cell or transgenic plant comprising a polynucleotide encoding a polypeptide tfdA which confers resistance to phenoxy auxin herbicide, such as 2-4-D. The reference also teaches an expression cassette comprising said polynucleotide operably linked to a 35S promoter or a plant, and wherein said expression cassette is used in obtaining said transgenic plant. See in particular, abstract; figures 1-7; columns 2-22.

The property of conferring resistance to a pyridyloxyl auxin herbicide would be inherent to the polynucleotide disclosed in the reference.

Kaphammer et al. anticipates the instantly claimed invention because the stringent conditions (see page 22 of specification) recited in claims 19-21, 30, 73, and 75 would allow hybridization of the polynucleotide taught by Kaphammer et al. The reference further teaches that said transgenic plant cell or plant would comprise more than one copy of the transgene (an additional herbicide resistant gene).

Thus Kaphammer et al. anticipated the claimed invention.

Claim 42 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Perlak et al. (PNAS, 88:3324-3328, 1991).

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach insect resistant gene.

Perlak et al. teach an insect resistant gene isolated from *Bacillus thuringiensis* and its expression in a transgenic plant to produce an insect resistant transgenic plant. See page 3324, abstract; page 3325, table 1; page 3327, figure 2, table 2.

It would have been obvious and within the scope of an ordinary skill in the art to introduce an insect resistant gene of Perlak et

Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

al. in Kaphammer et al. transgenic plants using any method of plant transformation including the one taught by Kaphammer et al. or Perlak. One of ordinary skill in the art would have been motivated to do so for the purposes of obtaining transgenic plants that are resistant to insects and herbicides.

Claim 43 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Kasuga et al. (Nature Biotechnology, 17:287-291, 1999).

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach a stress tolerance gene.

Kasuga et al. teach DREB1A gene overexpressed in a transgenic plant to produce drought, salt and freezing tolerant transgenic plant. See in particular, abstract; page 288, figures 1 and 2; page 289, figures 3-5.

It would have been obvious and within the scope of an ordinary skill in the art to introduce a stress tolerant gene of Kasuga et al. in Kaphammer et al. transgenic plants using any method of plant transformation including the one taught by Kaphammer et al. or Perlak. One of ordinary skill would have been motivated to do so for the purposes of obtaining transgenic plants that are resistant herbicides and exhibit tolerance to abiotic stresses.

Claim 77 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Verdaguer et al. (Plant Molecular Biology, 31:1129-1139, 1996)

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach a cassava vein mosaic virus promoter.

Verdaguer et al. teach a cassava vein mosaic virus promoter and its use in high level expression of a coding sequence of interest in a transgenic plant. See in particular, page 1129, abstract; pages 1130-1132, materials and methods.

It would have been obvious and within the scope of an ordinary skill in the art to replace the promoter used in expressing Kaphammer et al. polynucleotide in the transgenic plant with Verdaguer et al. cassava vein mosaic virus promoter. One of ordinary skill would have been motivated to do so for the purposes of obtaining transgenic plants expressing high levels of the transgenic protein, and thereby impart higher resistance to herbicides in said transgenic plants with reasonable expectation of success.

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) the claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers, claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

If a demand for international preliminary examination is made, the written opinion of the International Searching Authority will, except in certain cases where the International Preliminary Examining Authority did not act as International Searching Authority and where it has notified the International Bureau under Rule 66.1*bis*(b), be considered to be a written opinion of the International Preliminary Examining Authority. If a demand is made, the applicant may submit to the International Preliminary Examining Authority a reply to the written opinion together, where appropriate, with amendments before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later (Rule 43*bis*.1(c)).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see the *PCT Applicant's Guide*, Volume II.

Corteva Exhibit 2014 - Page 659 of 1122
Inari v. Corteva - PGR2023-00022

Electronic Patent Application Fee Transmittal	
Application Number:	12091896
Filing Date:	
Title of Invention:	Novel Herbicide Resistance Genes
First Named Inventor/Applicant Name:	Terry R. Wright
Filer:	Jay M. Sanders/Lisa Schodrowski
Attorney Docket Number:	DAS-129XC1
Filed as Large Entity	

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Oath/decl > 30 months from priority date	1617	1	130	130
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	130	130
Miscellaneous:				
		Total in USD ($)		**260**

Electronic Acknowledgement Receipt	
EFS ID:	4222763
Application Number:	12091896
International Application Number:	
Confirmation Number:	6428
Title of Invention:	Novel Herbicide Resistance Genes
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	23557
Filer:	Jay M. Sanders
Filer Authorized By:	
Attorney Docket Number:	DAS-129XC1
Receipt Date:	03-NOV-2008
Filing Date:	
Time Stamp:	16:21:24
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$260
RAM confirmation Number	1629
Deposit Account	020390
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	DAS_P0129_Petition.PDF	86496 852b2b76c8c3658c93f040eb4c1af3b64d75c014	no	1
Warnings:					
Information:					
2		DAS_p0129_Response.PDF	1013435 7ba0a19fa62570bd07eeebbaf1f6463061f3978f	yes	21

Multipart Description/PDF files in .zip description

Document Description	Start	End
Applicant Response to Pre-Exam Formalities Notice	1	3
Oath or Declaration filed	4	6
Foreign Reference	7	21

Warnings:					
Information:					
3	Fee Worksheet (PTO-06)	fee-info.pdf	32449 3ffbedbcf94c61a95576a6a29676ddafd813673c	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1132380		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/22 (01-08)
Approved for use through 06/30/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE
Under the paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless if displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) **FY 2008** *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) DAS-P0129-02-US (formerly DAS-129CX)
Application Number 12/9091,896	Filed April 28, 2008
For NOVEL HERBICIDE RESISTANCE GENES	
Art Unit Not Yet Assigned	Examiner Not yet assigned

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
[X]	One month (37 CFR 1.17(a)(1))	$120	$60	$ 120.00
[ ]	Two months (37 CFR 1.17(a)(2))	$460	$230	$
[ ]	Three months (37 CFR 1.17(a)(3))	$1050	$525	$
[ ]	Four months (37 CFR 1.17(a)(4))	$1640	$820	$
[ ]	Five months (37 CFR 1.17(a)(5))	$2230	$1115	$

[ ] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[ ] Payment by credit card. Form PTO-2038 is attached.

[ ] The Director has already been authorized to charge fees in this application to a Deposit Account.

[X] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 02-0390. I have enclosed a duplicate copy of this sheet.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

[X] attorney or agent of record. Registration Number 39,355

[ ] attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

Signature	November 3, 2008 Date
Jay M. Sanders Typed or printed name	317-237-0300 Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[ ] Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/SB/22 (01-08)
Approved for use through 06/30/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE
Under the paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless if displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) **FY 2008** (*Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).*)	Docket Number (Optional) DAS-P0129-02-US (formerly DAS-129CX)
Application Number 12/091,896	Filed April 28, 2008
For NOVEL HERBICIDE RESISTANCE GENES	
Art Unit Not Yet Assigned	Examiner Not yet assigned

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
[X]	One month (37 CFR 1.17(a)(1))	$120	$60	$ 120.00
[ ]	Two months (37 CFR 1.17(a)(2))	$460	$230	$
[ ]	Three months (37 CFR 1.17(a)(3))	$1050	$525	$
[ ]	Four months (37 CFR 1.17(a)(4))	$1640	$820	$
[ ]	Five months (37 CFR 1.17(a)(5))	$2230	$1115	$

[ ] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[ ] Payment by credit card. Form PTO-2038 is attached.

[ ] The Director has already been authorized to charge fees in this application to a Deposit Account.

[X] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 02-0390. I have enclosed a duplicate copy of this sheet.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

[X] attorney or agent of record. Registration Number 39,355

[ ] attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

November 3, 2008

Signature	Date
Jay M. Sanders	317-237-0300
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[ ] Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt	
EFS ID:	4223836
Application Number:	12091896
International Application Number:	
Confirmation Number:	6428
Title of Invention:	Novel Herbicide Resistance Genes
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	23557
Filer:	Jay M. Sanders/Lisa Schodrowski
Filer Authorized By:	Jay M. Sanders
Attorney Docket Number:	DAS-129XC1
Receipt Date:	03-NOV-2008
Filing Date:	
Time Stamp:	17:11:25
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	DAS_P0129_Petition.PDF	140974 d6b2e44cbeddeb13d727c241d82811003dd37ea6	no	1
Warnings:					
Information:					

Total Files Size (in bytes):	140974

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Print Form

PTO/SB/81 (11-08)
Approved for use through 11/30/2011. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

POWER OF ATTORNEY OR REVOCATION OF POWER OF ATTORNEY WITH A NEW POWER OF ATTORNEY AND CHANGE OF CORRESPONDENCE ADDRESS

Application Number	12/091,896
Filing Date	April 28, 2008
First Named Inventor	Terry R. Wright
Title	Novel Herbicide Resistance Genes
Art Unit	
Examiner Name	
Attorney Docket Number	DAS-P0129-US-02

I hereby revoke all previous powers of attorney given in the above-identified application.

☐ A Power of Attorney is submitted herewith.

OR

☒ I hereby appoint Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith:

83067

OR

☐ I hereby appoint Practitioner(s) named below as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith:

Practitioner(s) Name	Registration Number

Please recognize or change the correspondence address for the above-identified application to:

☒ The address associated with the above-mentioned Customer Number.

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the:

☐ Applicant/Inventor.

OR

☒ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) (Form PTO/SB/96) submitted herewith or filed on _____.

SIGNATURE of Applicant or Assignee of Record

Signature	Richard Bu	Date	1-15-09
Name	ANDREA T. BORUCKI	Telephone	317-337-4820
Title and Company	GENERAL PATENT COUNSEL, DOW AGROSCIENCES LLC		

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☒ *Total of 2 forms are submitted.

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/SB/96 (09-06)
Approved for use through 03/31/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: Dow AgroSciences LLC

Application No./Patent No.: 12/091,896 Filed/~~Issue~~ Date: April 28, 2008

Entitled:

Dow AgroSciences LLC, a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:
1. ☑ the assignee of the entire right, title, and interest; or

2. ☐ an assignee of less than the entire right, title and interest
(The extent (by percentage) of its ownership interest is _____%)

in the patent application/patent identified above by virtue of either:

A. ☑ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 021361, Frame 0768, or for which a copy thereof is attached.

OR

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet.

☐ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Signature	1-15-09 Date
Andrea T. Borucki Printed or Typed Name	(317) 337-4820 Telephone Number
General Patent Counsel Title	

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

5296574

Electronic Acknowledgement Receipt	
EFS ID:	4685544
Application Number:	12091896
International Application Number:	
Confirmation Number:	6428
Title of Invention:	Novel Herbicide Resistance Genes
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	23557
Filer:	Jay M. Sanders/Lisa Schodrowski
Filer Authorized By:	Jay M. Sanders
Attorney Docket Number:	DAS-129XC1
Receipt Date:	27-JAN-2009
Filing Date:	
Time Stamp:	16:49:10
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		DAS_P0129_POA.PDF	168885 7388d4ec585cc49225c1eaa1e95309f192033069	yes	2

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Power of Attorney	1	1
	CRF Statement Paper and CRF are the same	2	2
Warnings:			
Information:			
	Total Files Size (in bytes):	168885	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
12/091,896	Terry R. Wright	DAS-129XC1

83067
Baker & Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, ID 46204

INTERNATIONAL APPLICATION NO.	
PCT/US06/42133	
I.A. FILING DATE	PRIORITY DATE
10/27/2006	10/28/2005

CONFIRMATION NO. 6428
371 FORMALITIES LETTER

OC000000037481227

Date Mailed: 08/24/2009

NOTIFICATION OF DEFECTIVE RESPONSE

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as a Designated Office (37 CFR 1.494):

- Priority Document
- Copy of the International Application filed on 04/28/2008
- Copy of the International Search Report filed on 09/12/2008
- Copy of IPE Report filed on 08/23/2009
- Preliminary Amendments filed on 04/28/2008
- Biochemical Sequence Diskette filed on 04/28/2008
- Oath or Declaration filed on 11/03/2008
- Biochemical Sequence Listing filed on 04/28/2008
- U.S. Basic National Fees filed on 04/28/2008
- Priority Documents filed on 04/28/2008
- Power of Attorney filed on 01/27/2009
- Specification filed on 04/28/2008
- Claims filed on 04/28/2008
- Abstracts filed on 04/28/2008
- Drawings filed on 04/28/2008
- Paper nucleotide sequence listings filed on 04/28/2008

Applicant's response filed 08/23/2009 is hereby acknowledged. The following requirements set forth in the NOTIFICATION of MISSING REQUIREMENTS mailed 08/04/2008 have not been completed.

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date. The current oath or declaration does not comply with 37 CFR 1.497(a) and (b) in that it:
  - The name order of the fifth inventor is different than listed on the International application.

Applicant is required to complete the response within a time limit of ONE MONTH from the date of this Notification or within the time remaining in the response set forth in the Notification of Missing Requirements, whichever is the longer. No extension of this time limit may be granted under 37 CFR 1.136, but the period for response set in the Notification of Missing Requirements may be extended under 37 CFR 1.136(a).

FORM PCT/DO/EO/916 (371 Formalities Notice)

Corteva Exhibit 2014 - Page 672 of 1122
Inari v. Corteva - PGR2023-00022

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

CHARITTA A SHELTON

Telephone: (703) 756-1471

FORM PCT/DO/EO/916 (371 Formalities Notice)

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 08/24/2009

CBURT1 SALE #00000005 Mailroom Dt: 04/28/2008 190065 12091896
01 FC : 1641 100.00 DA

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 08/24/2009

CBURT1 ADJ #00000006 Mailroom Dt: 04/28/2008
Seq No: 15 Sales Acctg Dt: 08/04/2008 190065 12091896
01 FC : 1632 510.00 CR

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2006/042133	International filing date *(day/month/year)* 27 October 2006 (27.10.2006)	Priority date *(day/month/year)* 28 October 2005 (28.10.2005)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant DOW AGROSCIENCES LLC		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☒	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 17 March 2009 (17.03.2009)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Agnes Wittmann-Regis e-mail: pt06.pct@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX 142950
GAINESVILLE, FL 32614-2950

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	28 MAR 2008
Applicant's or agent's file reference DAS-129XC1	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US06/42133	27 October 2006 (27.10.2006)	28 October 2005 (28.10.2005)

International Patent Classification (IPC) or both national classification and IPC

IPC(8): **A01H 5/00**(2006.01);**C12N 15/82**(2006.01);**C12N 5/04**(2006.01)
USPC: 800/295;800/300;800/301;800/302;800/278;800/289;435/468;435/419

Applicant

DOW AGROSCIENCES LLC

1. This opinion contains indications relating to the following items:

- ☒ Box No. I — Basis of the opinion
- ☐ Box No. II — Priority
- ☐ Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV — Lack of unity of invention
- ☒ Box No. V — Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI — Certain documents cited
- ☐ Box No. VII — Certain defects in the international application
- ☒ Box No. VIII — Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(*b*) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	01 December 2007 (01.12.2007)	Vinod Kumar Telephone No. (571) 272-4445

Form PCT/ISA/237 (cover sheet) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☒ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☒ on paper

☒ in electronic form

c. time of filing/furnishing

☒ contained in the international application as filed.

☒ filed together with the international application in electronic form.

☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:
   - ☐ paid additional fees
   - ☐ paid additional fees under protest and, where applicable, the protest fee
   - ☐ paid additional fees under protest but the applicable protest fee was not paid
   - ☒ not paid additional fees
2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
   - ☐ complied with
   - ☒ not complied with for the following reasons:

   See the lack of unity section of the International Search Report(Form PCT/ISA/210)

4. Consequently, this opinion has been established in respect of the following parts of the international application:
   - ☐ all parts.
   - ☒ the parts relating to claims Nos. 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, and 83-94 (SEQ ID NOs: 1 and 2)

Form PCT/ISA/237 (Box No. IV) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO
Inventive step (IS)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO
Industrial applicability (IA)	Claims Please See Continuation Sheet	YES
	Claims Please See Continuation Sheet	NO

2. Citations and explanations:

Please See Continuation Sheet

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US06/42133

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 19-21, 30, 73, and 75 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims 19-21, 30, 73, and 75 not fully supported by the description. The application, as originally filed, did not describe genus of sequences that would hybridize to SEQ ID NO: 1. These claims encompass structures whose structures are not described in the specification, and thus one skilled in the art would not know their function. The stringent conditions described on page 22 of specification would encompass hybridization of unrelated nucleic acid sequences to instant SEQ ID NO: 1.

Claims 19-21, 30, 73, and 75 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because of the claims 19-21, 30, 73, and 75 are not fully supported by the description. The description does not disclose the claimed invention in a manner sufficiently clear and complete for the claimed invention to be carried out by a person skilled in the art because the stringent conditions described on page 22 of specification would encompass hybridization of unrelated nucleic acid sequences to instant SEQ ID NO: 1. One skilled in the art would not know how to use said unrelated sequences or plants having said unrelated sequences.

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Inari v. Corteva - PGR2023-00022

Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

V.1. Reasoned Statements:
The opinion as to Novelty was positive (Yes)with respect to claims 1-4, 42-43, 60-64, 77, 79-81, 83-84
The opinion as to Novelty was negative (No) with respect to claims 15-21, 30-32, 73-76
The opinion as to Inventive Step was positive (Yes)with respect to claims 1-4, 60-64, 79-81, 83-84
The opinion as to Inventive Step was negative(NO) with respect to claims 15-21, 30-32, 42-43, 73-77
The opinion as to Industrial Applicability was positive (YES) with respect to claims 1-4, 15-21, 30-32, 42-43, 60-64, 73-77, 79-81, 83-84
The opinion as to Industrial Applicability was negative(NO) with respect to claims NONE

V. 2. Citations and Explanations:

Claims 1-4, 42-43, 60-64, 77, 79-81, and 83-84 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a transgenic plant cell plant or seed derived thereof, comprising a polynucleotide that encodes an AAD-12 protein of SEQ ID NO: 2, or a variant of SEQ ID NO: 2 having aryloxyalkanoate dioxygenase activity and exhibiting at least 95% sequence identity to SEQ ID NO: 2. The prior art does not teach or failry suggest that said plant further comprises a gene for insect or stress resistance. The prior art does not teach or suggest an isolated polynucleotide that encodes a protein that enzymatically degrades a phenoxy auxin or pyridyloxy auxin herbicide, and wherein said polynucleotide is operably linked with a cassava vein mosaic virus promoter.

Claims 15-21, 30-32, 73-76 lack novelty under PCT Article 33(2) as being anticipated by Kaphammer et al. (US Patent No. 5,608,147, Issued March 4, 1997).

Kaphammer et al. teach a transgenic plant cell or transgenic plant comprising a polynucleotide encoding a polypeptide tfdA which confers resistance to phenoxy auxin herbicide, such as 2-4-D. The reference also teaches an expression cassette comprising said polynucleotide operably linked to a 35S promoter or a plant, and wherein said expression cassette is used in obtaining said transgenic plant. See in particular, abstract; figures 1-7; columns 2-22.

The property of conferring resistance to a pyridyloxyl auxin herbicide would be inherent to the polynucleotide disclosed in the reference.

Kaphammer et al. anticipates the instantly claimed invention because the stringent conditions (see page 22 of specification) recited in claims 19-21, 30, 73, and 75 would allow hybridization of the polynucleotide taught by Kaphammer et al. The reference further teaches that said transgenic plant cell or plant would comprise more than one copy of the transgene (an additional herbicide resistant gene).

Thus Kaphammer et al. anticipated the claimed invention.

Claim 42 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Perlak et al. (PNAS, 88:3324-3328, 1991).

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach insect resistant gene.

Perlak et al. teach an insect resistant gene isolated from *Bacillus thuringiensis* and its expression in a transgenic plant to produce an insect resistant transgenic plant. See page 3324, abstract; page 3325, table 1; page 3327, figure 2, table 2.

It would have been obvious and within the scope of an ordinary skill in the art to introduce an insect resistant gene of Perlak et

Corteva Exhibit 2014 - Page 682 of 1122
Inari v. Corteva - PGR2023-00022

Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

al. in Kaphammer et al. transgenic plants using any method of plant transformation including the one taught by Kaphammer et al. or Perlak. One of ordinary skill in the art would have been motivated to do so for the purposes of obtaining transgenic plants that are resistant to insects and herbicides.

Claim 43 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Kasuga et al. (Nature Biotechnology, 17:287-291, 1999).

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach a stress tolerance gene.

Kasuga et al. teach DREB1A gene overexpressed in a transgenic plant to produce drought, salt and freezing tolerant transgenic plant. See in particular, abstract; page 288, figures 1 and 2; page 289, figures 3-5.

It would have been obvious and within the scope of an ordinary skill in the art to introduce a stress tolerant gene of Kasuga et al. in Kaphammer et al. transgenic plants using any method of plant transformation including the one taught by Kaphammer et al. or Perlak. One of ordinary skill would have been motivated to do so for the purposes of obtaining transgenic plants that are resistant herbicides and exhibit tolerance to abiotic stresses.

Claim 77 lacks an inventive step under PCT Article 33(3) as being obvious over Kaphammer et al. in view of Verdaguer et al. (Plant Molecular Biology, 31:1129-1139, 1996)

Kaphammer et al. teachings are discussed above.

Kaphammer et al. do not teach a cassava vein mosaic virus promoter.

Verdaguer et al. teach a cassava vein mosaic virus promoter and its use in high level expression of a coding sequence of interest in a transgenic plant. See in particular, page 1129, abstract; pages 1130-1132, materials and methods.

It would have been obvious and within the scope of an ordinary skill in the art to replace the promoter used in expressing Kaphammer et al. polynucleotide in the transgenic plant with Verdaguer et al. cassava vein mosaic virus promoter. One of ordinary skill would have been motivated to do so for the purposes of obtaining transgenic plants expressing high levels of the transgenic protein, and thereby impart higher resistance to herbicides in said transgenic plants with reasonable expectation of success.

UNITED STATES NATIONAL STAGE WORKSHEET (DO/EO)

CHARITTA SHELTON, PAS

U. S. Application No. 12/091,896

Publication Date 5/10/2007 **WIPO number** 2007/053487

International App. # PCT/ US2006/042133 **Date** 10/27/2006

Publication language: English Priority doc. date 10/28/2005

Total Claims 15 **Chargeable claims** 15 **Independent claims** 2 **multiple claims** ___

371 Basic filing fee: ___ **insufficient** ___ SME fee paid ___ **Search fee US** ___ **IPER meets Art.33 (1)-(4) no fees** ___

Original claims replaced by Art.19 and/or Art.34 amendment ___

Total drawing sheets 3 foreign text not translated ___

Oath/Declaration: Unsigned ___ **Defective** ✓ **Completion date** ___

Biochemical Seq. Diskette ___ error report date ___ entered date ___

Biochemical Seq. listing ___ CRF statement in file ___

Copy of ISR 210/220 ___ references ___ Copy of ISA 237 ___ 373 ✓ IPER 409 ___

Art.34 annexes ___ not entered (see notes) ___ Art.19 Amendment ___

Preliminary Amendment ✓ used to calculate claim fee: yes ___ no ___ / 2nd Amendment dated ___

Information Disclosure Statement ___ / filed after original date ___ / 2nd IDS dated ___

Request for Immediate Examination: yes ___ **no** ✓

Substitute Specification ___ Assignment filed ___

Priority Doc. Present: ✓

Power of Attorney ✓ doc. dated 01-27-2009

PCT/RO/101 ___ U.S. Only: yes __ No _X_

Early publication request ADS ___ Petition to revive ___ Petition 1.47 ___

IB 306 filed for changes: ___ Other IB papers ___

---

35 USC Receipts of Request 4-28-08

Completion USC 371 Requirements ___

Notice of Missing Requirements (905) 8.2.2008

Notice of Acceptance (903) ___

Notice of regarding CRF Disk (922) ___

Notice of insufficient fees (923) ___

Notice of Defective Response (916) 23 August 2009

Notice of Abandonment (909) ___

--- **Notes:** The order of the fifth named inventor is different than the IA

I hereby certify that this correspondence is being electronically filed in the United States Patent and Trademark Office on Sept. 24, 2009

Jay M. Sanders, Patent Attorney

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors	Terry R. Wright et al	)	
Title:	NOVEL HERBICIDE RESISTANCE GENES	)	
Serial No.:	12/091,896	)	Confirmation No. 6428
Filing Date:	April 28, 2008	)	
Attorney Docket No.:	DAS-P0129-US-02 (formerly DAS-129XC1)	)	

RESPONSE TO NOTIFICATION OF DEFECTIVE RESPONSE

In response to the Notification of Defective Response mailed on August 24, 2009 (a copy of which is attached), Applicants submit that the name order of the fifth inventor as it appeared on the oath/declaration he signed (filed on November 3, 2008) is the most proper order of his names.

The order of his names appearing on the PCT Request was based in part on data mis-entry and possibly on a transliteration error.

The discrepancy between the name of the inventor indicated on the PCT Request and the name of the inventor as it appears on the oath/declaration was not due to a legal name change by court order or on account of marriage (the fifth inventor is a male), for example.

An updated Application Data Sheet accompanies this Response.

This Response is believed to be in accordance with the last paragraph of MPEP 1893.01(e), and 37 CFR 1.497(a) and (b) are believed to have been satisfied by the previously filed oath/declaration.

The Commissioner is hereby authorized to charge any fees that may be due in connection with this Response to Baker & Daniels LLP's Deposit Account No. 02-0390.

Respectfully submitted,

BAKER & DANIELS, LLP

Jay M. Sanders
Registration No. 39,355
Baker & Daniels LLP
300 North Meridian Street,
Suite 2700
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Telephone: (317) 237-1245
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Date Submitted: September 24, 2009

BDDB01 5854510v1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
12/091,896	Terry R. Wright	DAS-129XC1

83067
Baker & Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, ID 46204

INTERNATIONAL APPLICATION NO.	
PCT/US06/42133	
I.A. FILING DATE	PRIORITY DATE
10/27/2006	10/28/2005

RECEIVED
AUG 26 2009
BAKER & DANIELS LLP

CONFIRMATION NO. 6428
371 FORMALITIES LETTER



Date Mailed: 08/24/2009

NOTIFICATION OF DEFECTIVE RESPONSE

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as a Designated Office (37 CFR 1.494):

- Priority Document
- Copy of the International Application filed on 04/28/2008
- Copy of the International Search Report filed on 09/12/2008
- Copy of IPE Report filed on 08/23/2009
- Preliminary Amendments filed on 04/28/2008
- Biochemical Sequence Diskette filed on 04/28/2008
- Oath or Declaration filed on 11/03/2008
- Biochemical Sequence Listing filed on 04/28/2008
- U.S. Basic National Fees filed on 04/28/2008
- Priority Documents filed on 04/28/2008
- Power of Attorney filed on 01/27/2009
- Specification filed on 04/28/2008
- Claims filed on 04/28/2008
- Abstracts filed on 04/28/2008
- Drawings filed on 04/28/2008
- Paper nucleotide sequence listings filed on 04/28/2008

Missing Part
REPLY DUE: 9-24-09
DOCKET 1 CP DOCKET 2

Applicant's response filed 08/23/2009 is hereby acknowledged. The following requirements set forth in the NOTIFICATION of MISSING REQUIREMENTS mailed 08/04/2008 have not been completed.

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date. The current oath or declaration does not comply with 37 CFR 1.497(a) and (b) in that it:
  - The name order of the fifth inventor is different than listed on the International application.

Applicant is required to complete the response within a time limit of ONE MONTH from the date of this Notification or within the time remaining in the response set forth in the Notification of Missing Requirements, whichever is the longer. No extension of this time limit may be granted under 37 CFR 1.136, but the period for response set in the Notification of Missing Requirements may be extended under 37 CFR 1.136(a).

FORM PCT/DO/EO/916 (371 Formalities Notice)

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

CHARITTA A SHELTON

Telephone: (703) 756-1471

FORM PCT/DO/EO/916 (371 Formalities Notice)

SEQUENCE LISTINGS AND TABLES RELATED THERETO IN INTERNATIONAL APPLICATIONS FILED IN THE U.S. RECEIVING OFFICE

The Administrative Instructions (AIs) under the Patent Cooperation Treaty (PCT), in force as of **July 1, 2009**, contain important changes relating to the manner of filing, and applicable fees for, sequence listings and/or tables related thereto (sequence-related tables) in international applications. The complete text may be accessed at http://www.wipo.int/pct/en/texts/index.htm.

Effective July 1, 2009, Part 8 and Annex C-*bis* will no longer form part of the AIs. Part 8 was introduced in 2001 as a temporary solution to problems arising from the filing of very large sequence listings on paper and provided for a *sequence listing forming part of the international application* to be filed in electronic form on physical medium (*e.g.*, CD), together with the remainder of the application on paper. In 2002, Part 8 was expanded to include sequence-related tables and Annex C-*bis* was added to provide technical requirements. All applicants may now file complete international applications in electronic form, eliminating the need for these temporary provisions.

I. AIS PART 8 AND ANNEX C-*BIS DELETED AS OF JULY 1, 2009*

A) **Sequence-related tables cannot be filed as a separate part of the description or in text format.** They must be provided as an integral part of the international application either:
- in PDF format as part of an international application filed in electronic form via EFS-Web; or
- on paper as part of an international application filed on paper.

B) A ***sequence listing forming part of an international application*** may be provided either:
- in electronic form, as part of an international application filed in electronic form via EFS-Web, in
  - Annex C/ST.25 text format (preferred), or
  - PDF format; or
- on paper as part of an international application filed on paper.

C) A ***sequence listing not forming part of the international application*** **(for search under PCT Rule 13*ter*) in Annex C/ST.25 text format**
- is not required where the *sequence listing forming part of the international application* was filed in Annex C/ST.25 text format as part of an international application filed in electronic form via EFS-Web
- is required for search where the *sequence listing forming part of the international application* was filed in PDF
- is required for search on physical medium (*e.g.*, CD) where the *sequence listing forming part of the international application* was filed on paper as part of an international application filed on paper.

II. CALCULATION OF THE INTERNATIONAL FILING FEE AND FEE REDUCTION UNDER AI § 707

A) **A sequence-related table** must form an integral part of the international application and will incur FULL page fees with no upper limit.

B) A ***sequence listing forming part of an international application*** filed:
- via EFS-Web in Annex C/ST.25 text format will incur NO page fees;
- on paper or in PDF format will incur FULL page fees with no upper limit.

III. AVAILABILITY OF SEQUENCE LISTINGS SUBMITTED FOR SEARCH UNDER PCT RULE *13TER*

International Searching Authorities will be required to transmit to the International Bureau a copy of an Annex C/ST.25 text format sequence listing provided for search under PCT Rule 13*ter*. Any such sequence listing will be made available on PATENTSCOPE® (*sequence listings forming part of the international application* are already available).

IV. JULY 2009 REQUEST (PCT/RO/101)

The Request now has two options for the last sheet: one for paper filings; and one for EFS-Web filings. The July 2009 Request may be accessed at http://www.wipo.int/pct/en/forms/index.htm.

Application Information

Application Type::	Regular (National Stage)
Subject Matter::	Utility
Suggested Classification::	None
Suggested Group Art Unit::	None
CD-ROM or CD-R?::	None
Number of CD disks::	None
Number of copies of CDs::	None
Sequence submission?::	Paper
Computer Readable Form?::	Yes
Number of Copies of CRF::	None
Title::	NOVEL HERBICIDE RESISTANCE GENES
Attorney Docket Number::	DAS-P0129-US-02
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	None
Total Drawing Sheets::	3
Small Entity?::	No
Petition included?::	No
Petition Type::	N/A
Secrecy Order in Parent Appl.?::	No

Corteva Exhibit 2014 - Page 689 of 1122
Inari v. Corteva - PGR2023-00022

Applicant Information

Applicant Authority Type::	Inventor
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Applicant Information

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State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46038

Corteva Exhibit 2014 - Page 690 of 1122
Inari v. Corteva - PGR2023-00022

Applicant Information

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Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46032

Corteva Exhibit 2014 - Page 691 of 1122
Inari v. Corteva - PGR2023-00022

Applicant Information

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Postal or Zip Code of Mailing Address::	46032

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Primary Citizenship Country::	US
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State or Province of Residence::	IN
Country of Residence::	US
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City of Mailing Address::	Zionsville
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46077

Corteva Exhibit 2014 - Page 692 of 1122
Inari v. Corteva - PGR2023-00022

Representative Information

Representative Customer Number::	000083067

Correspondence Information

Correspondence Customer Number::	000083067
Telephone Number One::	(317) 237-1245
Telephone Number Two::	
Fax Number::	(317) 237-1000
Electronic Mail Address::	jay.sanders@bakerd.com

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This application is a	National Stage of	PCT/US2006/042133	October 27, 2006
PCT/US2006/042133	An application claiming the benefit under 35 USC 119(e) of	60/731,044	October 28, 2005

Assignee Information

Assignee Name::	DOW AGROSCIENCES, LLC
Street of Mailing Address::	9330 Zionsville Road
City of Mailing Address::	Indianapolis
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46268-1054

Corteva Exhibit 2014 - Page 693 of 1122
Inari v. Corteva - PGR2023-00022

UNITED STATES NATIONAL STAGE WORKSHEET (DO/EO)

CHARITTA SHELTON, PAS

U. S. Application No. 12/091,896

Publication Date 5/10/2007 WIPO number 2007/053487

International App. # PCT/US2006/042133 Date 10/27/2006

Publication language: English Priority doc. date 10/28/2005

Total Claims 15 Chargeable claims 15 Independent claims 2 multiple claims ___

371 Basic filing fee: ___ insufficient ___ SME fee paid ___ Search fee US ___ IPER meets Art.33 (1)-(4) no fees ___

Original claims replaced by Art.19 and/or Art.34 amendment ___

Total drawing sheets 3 foreign text not translated ___

Oath/Declaration: Unsigned ___ Defective ✓ Completion date ___

Biochemical Seq. Diskette ___ error report date ___ entered date ___

Biochemical Seq. listing ___ CRF statement in file ___

Copy of ISR 210/220 ___ references ___ Copy of ISA 237 ___ 373 ✓ IPER 409 ___

Art.34 annexes ___ not entered (see notes) ___ Art.19 Amendment ___

Preliminary Amendment ✓ used to calculate claim fee: yes ___ no ___ / 2nd Amendment dated ___

Information Disclosure Statement ___ / filed after original date ___ / 2nd IDS dated ___

Request for Immediate Examination: yes ___ no ✓

Substitute Specification ___ Assignment filed ___

Priority Doc. Present: ✓

Power of Attorney ✓ doc. dated 01-27-2009

PCT/RO/101 ___ U.S. Only: yes __ No _X_

Early publication request ADS ___ Petition to revive ___ Petition 1.47 ___

IB 306 filed for changes: ___ Other IB papers ___

---

35 USC Receipts of Request 4-28-08

Completion USC 371 Requirements ___

Notice of Missing Requirements (905) 8.2.2008

Notice of Acceptance (903) ___

Notice of regarding CRF Disk (922) ___

Notice of insufficient fees (923) ___

Notice of Defective Response (916) 23 August 2009

Notice of Abandonment (909) ___

--- Notes: The order of the fifth named inventor is different than the IA

Electronic Acknowledgement Receipt	
EFS ID:	6138010
Application Number:	12091896
International Application Number:	
Confirmation Number:	6428
Title of Invention:	Novel Herbicide Resistance Genes
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	83067
Filer:	Jay M. Sanders
Filer Authorized By:	
Attorney Docket Number:	DAS-129XC1
Receipt Date:	24-SEP-2009
Filing Date:	
Time Stamp:	16:10:12
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		DAS_P0129_Response_Notification.PDF	373720 5c071d90e3da0dbb86b02b2459bf0e426d8a82f7	yes	10

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Applicant Response to Pre-Exam Formalities Notice	1	3
	Application Data Sheet	4	10
Warnings:			
Information:			
	Total Files Size (in bytes):	373720	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO SB08

Substitute for form 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT *(Use as many sheets as necessary)*				Application Number	12/091,896
				Filing Date	April 28, 2008
				First Named Inventor	Terry R. Wright, et al.
				Art Unit	1616
				Examiner Name	Not Assigned
Sheet	1	of	2	Attorney Docket Number	DAS-P0129-US-02

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	Cite No.	DOCUMENT NUMBER Number-Kind Code (if known)	Publication Date MM-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA	US-5,608,147	03-1997	Bryan J. Kaphammer	All
	AB				
	AC				
	AD				
	AE				
	AF				
	AG				
	AH				
	AI				
	AJ				
	AK				
	AL				
	AM				
	AN				
	AO				
	AP				
	AQ				
	AR				
	AS				

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.	Foreign Patent Document Country Code-Number-Kind Code (if known)	Publication Date MM-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	Translation
	AT	WO 2005/107437	11-2005	Wright, et al.	All	
	AU					
	AV					
	AW					
	AX					
	AY					

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

BDDB01 6203502v1

Substitute for form 1449/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT *(Use as many sheets as necessary)*				Application Number	12/091,896
				Filing Date	April 28, 2008
				First Named Inventor	Terry R. Wright, et al.
				Art Unit	1616
				Examiner Name	Not Assigned
Sheet	2	of	2	Attorney Docket Number	DAS-P0129-US-02

NON PATENT LITERATURE DOCUMENTS		
*EXAMINER INITIAL	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.
	BA	SCHLEINITZ, et al., Localization and Characterization of two Novel Genes Encoding Stereospecific Diosygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in *Delftia acidovorans* MC1, Applied and Environmental Michrobiology, Sept. 2004, p. 5357-5365
	BB	GRESSEL, Jonathan, Molecular biology of week control, Transgenic Research 9, p. 355-382, 2000
	BC	HEGG, Eric L. et al., Herbicide-Degrading α-Keto Acid-Dependent Enzyme TfdA: Metal Coordination Environment and Mechanistic Insights, Biochemistry, 1999, 38, p 16714-16726
	BD	PERLAK, Frederick J., Modification of the Coding Sequence Enhances Plant Expression of Insect Control Protein Genes, *Proc. Natl., Acad. Sci.*, USA, Biochemistry, Vol. 88, pp. 3324-3328, April 1991
	BE	KASUGA, Mie, Improving Plant Drought, Saqlt, and Freezing Tolerance by Gene Transfer of a Single Stress-Inducible Transcription Factor, Nature Biotechnology, March 1999, Vol. 17, pp. 287-291
	BF	VERDAGUER, Bertrand, Isolation and Expression in Transgenic Tobacco and Rice Plants, of the Cassava Vein Mosaic Virus (CVMV) Promoter, Plant Molecular Biology 31, 1996, pp. 1129-1139
	BG	
	BH	
	BI	
	BJ	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

BDDB01 6203502v1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	:	1616
Applicants	:	Terry R. Wright, Justin M. Lira, Terence A. Walsh, Donald J. Merlo, Pon Samuel Jayakumar, Gaofeng Lin.
Serial No.	:	12/091,896
Conf. No.	:	6428
Filed	:	April 28, 2008.
For	:	Novel herbicide resistance genes

INFORMATION DISCLOSURE STATEMENT

This statement is filed, in the application identified above, pursuant to 37 C.F.R. §§ 1.56, 1.97, and 1.98.

Attached hereto is a copy of the Form PTO SB/08 and copies of the references (except for the U.S. patent) listed thereon.

Respectfully submitted,

Jay M. Sanders
Registration No. 39,355
Phone No.: 317 237 1245
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Address: 300 N. Meridian Street
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Indianapolis, IN 46204

JMS/jlk

Date Submitted: July 20, 2010

Attachments

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 November 2005 (17.11.2005)

PCT

(10) International Publication Number
WO 2005/107437 A2

(51) International Patent Classification[7]: A01H 5/00

(21) International Application Number:
PCT/US2005/014737

(22) International Filing Date: 2 May 2005 (02.05.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/567,052 30 April 2004 (30.04.2004) US

(71) Applicant *(for all designated States except US)*: **DOW AGROSCIENCES LLC** [US/US]; 9330 Zionsville Road, Indianapolis, IN 45268-1054 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WRIGHT, Terry, R.** [US/US]; 14162 Charity Chase Circle, Westfield, IN 46074 (US). **LIRA, Justin, M.** [US/US]; 12373 Ensley Drive, Fishers, IN 46038 (US). **MERLO, Donald, J.** [US/US]; 11845 Durbin Drive, Carmel, IN 46032 (US). **HOPKINS, Nicole** [US/US]; 2518 Chaseway Ct., Indianapolis, IN 46268 (US).

(74) Agents: **SANDERS, Jay, M.** et al.; Saliwanchik, Lloyd & Saliwanchik, A Professional Association, P.O. Box 142950, Gainesville, FL 32614-2950 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2005/107437 A2

(54) Title: NOVEL HERBICIDE RESISTANCE GENES

(57) Abstract: The subject invention provides novel plants that are not only resistant to 2,4-D and other phenoxy auxin herbicides, but also to aryloxyphenoxypropionate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention alone or "stacked" together with another herbicide resistance gene, preferably a glyphosate. resistance gene, so as to provide broader and more robust weed control, increased treatment flexibility, and improved herbicide resistance management options. More specifically, preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD* (aryloxyalkanoate dioxygenase) genes and proteins. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. This highly novel discovery is the basis of significant herbicide tolerant crop trait opportunities as well as development of selectable marker technology. The subject invention also includes related methods of controlling weeds. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the formation of, and controlling, weeds that are resistant (or naturally more tolerant) to one or more herbicides such as glyphosate.

NOVEL HERBICIDE RESISTANCE GENES

Cross-Reference to Related Application

[0001] This application claims priority to U.S. provisional application Serial No. 60/567,052, filed April 30, 2004.

Background of the Invention

[0002] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0003] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

[0004] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (*e.g.,* wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (*e.g.*, alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; *see* "isb.vt.edu/cfdocs/fieldtests1.cfm , 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0005] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has

been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

[0006] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Ng *et al.*, 2003; Simarmata *et al.*, 2003; Lorraine-Colwill *et al.*, 2003; Sfiligoj, 2004; Miller *et al.*, 2003; Heap, 2005; Murphy *et al.*, 2002; Martin *et al.*, 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis,* and *Plantago lanceolata.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0007] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting

no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

[0008] 2,4-D is in the phenoxy acid class of herbicides, as are MCPA, mecoprop, and dichlorprop. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

[0009] 2,4-D has different levels of selectivity on certain plants (*e.g.,* dicots are more sensitive than grasses). Differential metabolism of 2,4-D by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

[0010] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D is currently formulated to have a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0011] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0012] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many

homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with α-ketoglutarate dioxygenase Fe^{+2} dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

[0013] One unique example with low homology to *tfdA* (28% amino acid identity) is *rdpA* from *Sphingobium herbicidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organisms that degrade phenoxypropionic acid were described some time ago, little progress had been made in characterizing this pathway until recently (Horvath *et al.*, 1990). An additional complication to dichlorprop degradation is the stereospecificity (R *vs.* S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes, or transformation of this gene into plants, has not heretofore been reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids (*e.g.*, 2,4-D).

[0014] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0015] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and dichlorprop), pyridyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. Discovery of a multifunctional enzyme for the degradation of herbicides covering multiple modes would be both unique and valuable as an HTC trait.

Brief Summary of the Invention

[0016] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0017] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, AOPP, and one or more commercially available herbicides (*e.g.,* glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0018] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and AOPP herbicides, in an enantiospecific manner. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate

Dioxygenase). This highly novel discovery is the basis of significant HTC trait and selectable marker opportunities.

[0019] There was no prior motivation to produce plants comprising an *AAD-1* gene (preferably an *AAD-1* polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-1 enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides (such as quizalofop, haloxyfop, *et al.*). Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0020] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoate auxin herbicides, and aryloxyphenoxypropionate herbicides by a recombinantly expressed AAD-1 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more AOPP, phenoxy auxin, or other aryloxyalkanoate herbicides to plants comprising an *AAD-1* gene. The subject invention also provides methods of using an *AAD-1* gene as a selectable marker for identifying plant cells and whole plants transformed with *AAD-1*, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

Brief Description of the Figures

[0021] **Figure 1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

[0022] **Figure 2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

[0023] **Figure 3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

[0024] **Figure 4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

[0025] **Figure 5** shows 2,4-dichlorophenol production by recombinant *AAD-1*.

[0026] **Figures 6A and 6B** show phenol production by recombinant *AAD-1* from various herbicide substrates.

[0027] **Figure 7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

[0028] **Figures 8A and 8B** show that *AAD-1 (v3)* was expressed equally in *Arabidopsis* leaves of different ages but continued to accumulate throughout the 25 days of experiment. Plants that were not sprayed with the herbicide 2,4-D (panel A) expressed a little more AAD-1 (v3) than those had been sprayed (panel B). Bars represent the mean ± SEM of 5 leaves from 5 different plants, with percent expression of AAD-1 (v3) normalized to total soluble protein. Light bars represent the third young leaves (N-3) collected from the top, dark bars represent the 5th oldest leaves from the bottom.

[0029] **Figures 9A, 9B, and 9C** show injury of *Arabidopsis* plants after 2,4-D treatment. Four different lines were each treated with four different doses of 2,4-D and their injury was graded 4 (panel A) and 14 (panel B) days after treatment. Their expression of AAD-1 (v3) in leaves was also determined using ELISA (panel C). The results were mean ± SEM of five leaves from five different plants received the same treatment.

[0030] **Figure 10** illustrates that pDAB3230-transformed *Arabidopsis* (*AAD-1* + *EPSPS*) shows >14-fold level of glyphosate tolerance 7 DAT vs. wildtype and transformed control *Arabidopsis* lines.

[0031] **Figure 11** shows dose response of callused maize suspensions to R-haloxyfop.

[0032] **Figure 12** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol.

[0033] **Figure 13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

[0034] **Figure 14** shows the responses of several *AAD-1 (v3)*-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied as a postemergence spray 1 week prior.

[0035] **Figure 15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to

quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

[0036] **Figure 16** shows *AAD-1 (v3)*-transformed corn tolerant to 8X field rates of quizalofop (Assure II) under field conditions.

[0037] **Figure 17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

[0038] **Figure 18** shows Western Blotting analysis on soybean calli transformed with *AAD-1 (V3)* gene indicating that the callus cells are expressing AAD-1 (v3) protein.

[0039] **Figure 19** shows fitted curves for 2,4-D degradation rates by AAD-2 (v1) vs. AAD-1 (v1).

[0040] **Figure 20** shows the response of *AAD-1 v3* (plant optimized), or *AAD-1 (v2)* (native), *AAD-2 (v1)* (native), or *AAD-2 (v2)* (plant optimized)-transformed T_1 *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T_1 family.

[0041] **Figure 21** shows western blot analysis of individual native *AAD-2 (v1)*-transformed T_1 *Arabidopsis* plants. This shows that plants expressing the AAD-2 (v1) protein are suffering severe injury from 200 g ae (acid equivalent)/ha 2,4-D treatments, which normally causes little injury to native *AAD-1 (v2)* or plant optimized *AAD-1 (v3)*-transformed *Arabidopsis*. AAD-2 protein is identified on the gel. Several background bands were detected in *AAD-2*-transformed and *Pat/Cry1F*-transformed samples.

[0042] **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop.

Brief Description of the Sequences

[0043] **SEQ ID NO:1** is the sequence of a forward primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0044] **SEQ ID NO:2** is the sequence of a reverse primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0045] **SEQ ID NO:3** is the nucleotide sequence of *AAD-1 (v1)* from *Sphingobium herbicidovorans*.

[0046] **SEQ ID NO:4** is the nucleic acid sequence of the native *AAD-1* gene with internal NotI restriction site removed. This gene is designated *AAD-1 (v2)*. DNA sequencing

confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

[0047] **SEQ ID NO:5** is a "plant-optimized" DNA sequence *AAD-1 (v3)*. This "gene" encodes SEQ ID NO:11, which is the same as SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations.

[0048] **SEQ ID NO:6** ("rdpA(ncoI)") and **SEQ ID NO:7** ("3'saci") were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[0049] **SEQ ID NO:8** is another PCR primer ("BstEII/Del NotI") that was used with the "3' SacI" primer.

[0050] **SEQ ID NO:9** is the native amino acid sequence encoded by the *AAD-1 (v1)* gene from *Sphingobium herbicidovorans*.

[0051] **SEQ ID NO:10** is the amino acid sequence encoded by the *AAD-1 (v2)* DNA sequence of SEQ ID NO:4.

[0052] **SEQ ID NO:11** is the amino acid sequence encoded by the *AAD-1 (v3)* plant-optimized DNA sequence of SEQ ID NO:5.

[0053] **SEQ ID NO:12** is the DNA sequence of the native *AAD-2 (v1)* gene.

[0054] **SEQ ID NO:13** is the amino acid sequence of the AAD-2 (v1) protein.

[0055] **SEQ ID NO:14** is a forward primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0056] **SEQ ID NO:15** is a reverse primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0057] **SEQ ID NO:16** is the M13 forward primer.

[0058] **SEQ ID NO:17** is the M13 reverse primer.

[0059] **SEQ ID NO:18** is a forward primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0060] **SEQ ID NO:19** is a reverse primer used to amplify *AAD-2 (v1)* DNA for cloning.

[0061] **SEQ ID NO:20** is the native soybean EPSPS protein.

[0062] **SEQ ID NO:21** is a doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine).

[0063] **SEQ ID NO:22** is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

[0064] **SEQ ID NO:23** is primer Pat 5-3.

[0065] **SEQ ID NO:24** is primer Pat 3-3.

[0066] **SEQ ID NO:25** is forward primer AAD-1 PTU.

[0067] **SEQ ID NO:26** is reverse primer AAD-1 PTU.

[0068] **SEQ ID NO:27** is the forward primer for the Coding Region PCR AAD-1.

[0069] **SEQ ID NO:28** is the reverse primer for the Coding Region PCR AAD-1.

[0070] **SEQ ID NO:29** is the *AAD-2 (v2)* nucleotide (plant optimized).

[0071] **SEQ ID NO:30** is the translated AAD-2 (v2) protein sequence.

[0072] **SEQ ID NO:31** is the Southern fragment PCR AAD-1 forward primer.

[0073] **SEQ ID NO:32** is the Southern fragment PCR AAD-1 reverse primer.

Detailed Description of the Invention

[0074] The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of an herbicide tolerance trait for 2,4-D would be its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism and the like. An additional benefit of the *AAD-1* gene is that unlike all *tfdA* homologues characterized to date, AAD-1 is able to degrade the R-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (*e.g.*, dichlorprop and mecoprop) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). *See* **Table 1**. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the *AAD-1* gene in plants would afford protection to a much wider spectrum of phenoxy auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled, protecting from drift or other off-site phenoxy herbicide injury for the full breadth of commercially available phenoxy auxins.

[0075] **Table 1.** Commercially available phenoxy auxins. Reference to phenoxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as

substrates for AAD-1 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Table 1. Commercially available phenoxy auxins.

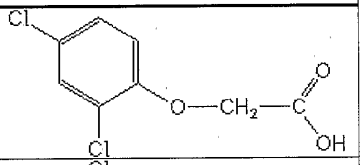
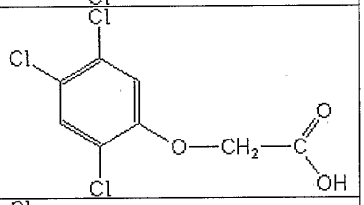
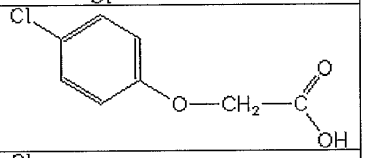
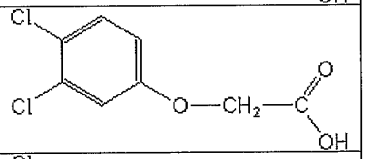
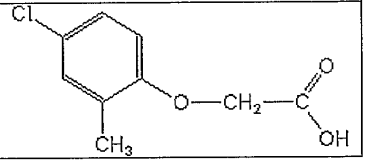
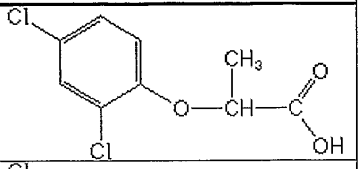
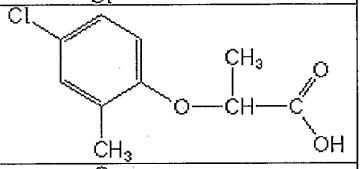
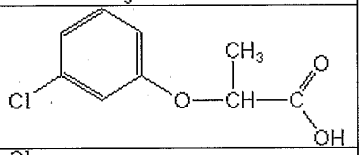
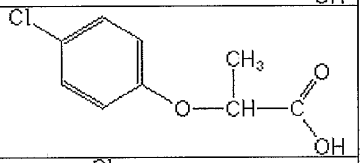
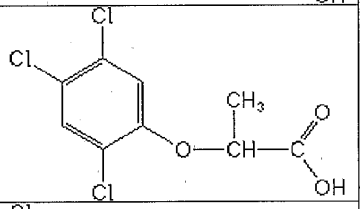
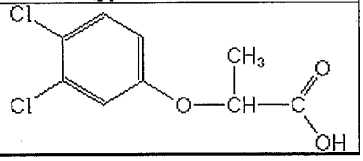
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	
MCPA	94-74-6	25 – 4000	125 - 1550	

Table 1. Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Dichlorprop	120-36-5	25 – 12000	100 - 2240	
Mecoprop	7085-19-0	25 – 4000	250 - 3360	
Cloprop	101-10-0	25 – 4000	25 – 4000	
4-CPP	3307-39-9	25 – 4000	25 – 4000	
Fenoprop	93-72-1	25 – 4000	25 – 4000	
3,4-DP	3307-41-3	25 – 4000	25 – 4000	

[0076] An additional benefit of the *AAD-1* gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). *See* **Table 2**. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing *AAD-1*, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, sorghum, warm and cool-

season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

[0077] **Table 2.** AOPP graminicidal compounds listed by accepted common names. Reference to AOPP herbicides is generally made to the active acid but most are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Table 2. AOPP graminicidal compounds listed by accepted common names.

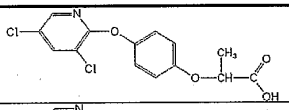
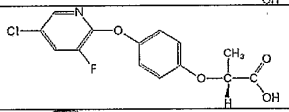
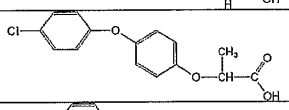
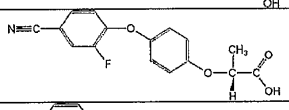
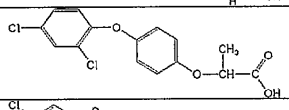
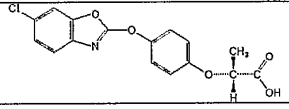
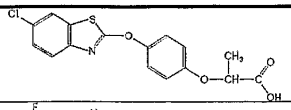
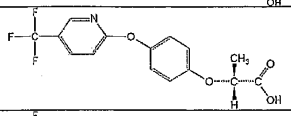
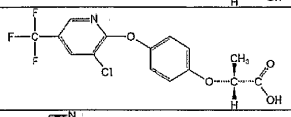
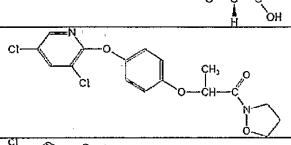
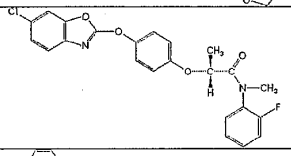
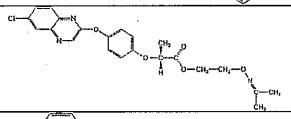
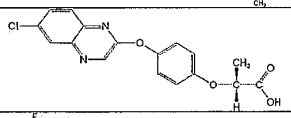
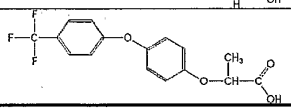
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	

Table 2. AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop	256412-89-2	10-2000	35-280	
Propaquizafop	111479-05-1	10-2000	30-240	
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

[0078] A single gene (*AAD-1*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-1* can provide protection *in planta* to AOPP herbicides where natural tolerance also was not sufficient to allow selectivity. Plants containing *AAD-1* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a

broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combinations of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

[0079] Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking *AAD-1* with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant weed species (either grass weed species with one or more AOPP herbicides, or broadleaf weed species with one or more phenoxy auxins) in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (*e.g.*, 2,4-D) and AOPP herbicide(s) (*e.g.*, quizalofop) selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from 2 hours to 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

[0080] It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/*AAD-1* stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and

environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0081] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0082] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant species or resistant weed species could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS, GOX, GAT*), glufosinate resistance (*e.g.*, *Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g.*, *PPO-1*), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), dicamba-degrading enzymes (*see, e.g.*, US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

[0083] Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0084] Additionally, *AAD-1* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress

tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0085] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes.

[0086] More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also AOPP herbicides. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase) genes and proteins.

[0087] This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate herbicides by a recombinantly expressed AAD-1 enzyme.

[0088] The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical and biological assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP (ranging from 50-100% conversion) *in vitro*. An additional advantage that *AAD-1*–transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0089] The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an *AAD-1* gene.

[0090] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0091] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0092] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0093] *RdpA*, from *Sphingobium herbicidovorans* (Westendorf *et al.*, 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath *et al.*,

1990). An additional complication to dichlorprop degradation is the stereospecificity (R *vs.* S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that *rdpA* or *AAD-1* genes could be successfully expressed in plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

[0094] As described in more detail in the Examples below, *rdpA* was cloned from *Sphingobium herbicidovorans* and tested for substrate promiscuity among various herbicide chemical classes. This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. *RdpA* has never been expressed in plants, nor was there any motivation to do so because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0095] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance are the aryloxyphenoxypropionate (AOPP) grass herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities.

[0096] The broad spectrum grass AOPP herbicides are reported herein to be excellent substrates for AAD-1 as well as 2,4-D, dichlorprop, and other phenoxy auxins. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxy auxins) and a range of broad spectrum, highly active grass herbicides (AOPPs).

[0097] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxy-

alkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

[0098] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf and grass weeds. *AAD-1* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (*e.g.*, glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, *et al.*), and insect resistance traits (*Cry1F, Cry1Ab, Cry 34/45, et al.*) for example. Additionally, *AAD-1* can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0099] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-1*-containing constructs and have demonstrated high levels of resistance to both the phenoxy auxin and AOPP herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention. As shown below in Example 6, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

[00100] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-1. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[00101] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

[00102] Additionally, glyphosate tolerant crops grown worldwide are prevalent. Many times in rotation with other glyphosate tolerant crops, control of glyphosate-resistant volunteers may be difficult in rotational crops. Thus, the use of the subject transgenic traits, stacked or transformed individually into crops, provides a tool for controlling other HTC volunteer crops.

[00103] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[00104] The transgenic expression of the subject *AAD-1* genes is exemplified in, for example, *Arabidopsis*, corn (maize), tobacco, cotton, rice, soybean, and canola. However, the subject invention can be applied to any other desired types of plants. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be applied to multiple other grass and other broadleaf crops. Likewise, 2,4-D can be more positively utilized in grass crops where tolerance to 2,4-D is moderate, and increased tolerance via this trait would provide growers the opportunity to use 2,4-D at more efficacious rates and over a wider application timing without the risk of crop injury.

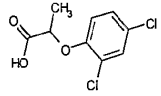
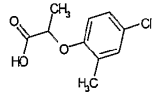
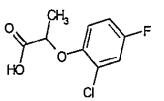
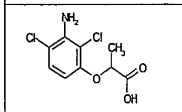
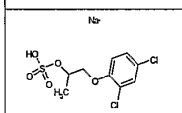
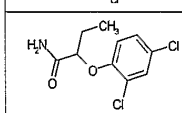
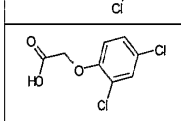
[00105] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed (auxins) and grass weeds (AOPPs). This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-1 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

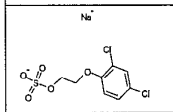
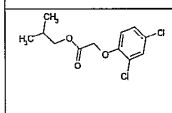
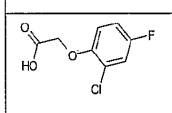
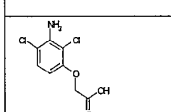
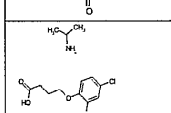
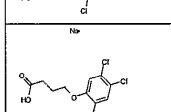
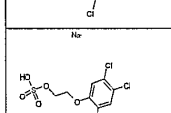
[00106] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

[00107] One gene of the subject invention, referred to herein as *AAD-1* (aryloxyalkanoate dioxygenase), was cloned from *Sphingobium herbicidovorans* (ATCC 700291) by PCR into pET 280-S/S (designated pDAB 3203) and expressed in BL-21 Star *E. coli.* When this gene is overexpressed (by induction of 1mM IPTG and culture lysate combined with the following reaction mix: 112.5 µg/ml 2,4-D, 1 mM Ascorbic acid, 1 mM α-ketoglutarate, 50 µM $Fe(NH_4)_2(SO_4)_2$, the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay). Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See **Tables 3 and 4**).

[00108] **Table 3:** Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Table 3: Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

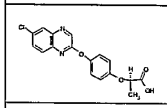
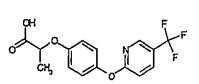
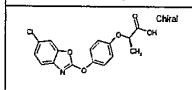
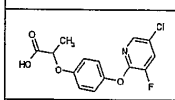
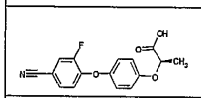
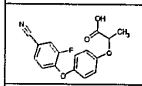
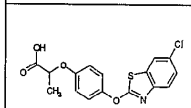
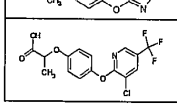
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383

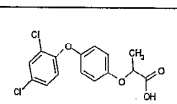
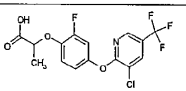
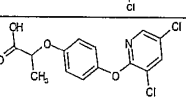
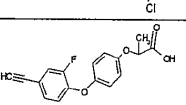
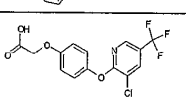
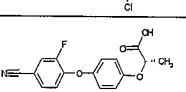
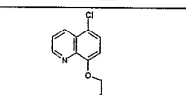
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
	178587		0.003	0.02
	188527		0.003	0.036

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[00109] **Table 4:** Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Table 4. Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalofop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17
	11044492	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinofop	0.295	1.98
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	460511	(R,S)-diclofop	0.155	1.663
	25646		0.144	1.69
	70222	(R,S)-chlorazifop	0.128	1.584
	199608	cyhalofop	0.114	1.26
	43865	haloxyfop-oxyacetate	0.004	0.053
	7466	(S)-cyhalofop	0.003	0.017
	204558	Cloquintocet	0	0.001

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[00110] Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By "functional activity" (or "active") it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce "an effective amount" of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[00111] Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. Optimized polynucleotide can also be designed based on the protein sequence.

[00112] The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe

or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

[00113] There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

[00114] One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria. Other members of the orders Firmicutes and Proteobacteria, and specific genera with known *rdpA*'s, such as *Sphingobium*, *Delftia*, *Rodoferax*, and *Comamonas* for example, can be used as source isolates.

[00115] Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutants can be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

[00116] A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example),

and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

[00117] Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook *et al.*, 1989, which is incorporated herein by reference.

[00118] Polynucleotides and probes. The subject invention further provides nucleotide sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

[00119] The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the *AAD-1* gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage.

[00120] As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense

strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

[00121] In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

[00122] Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

[00123] Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, *etc.*).

[00124] As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G.H., M.M. Manak (1987) *DNA Probes*, Stockton Press, New York, NY, pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2x SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1x SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1x SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2x SSC/0.1% SDS can be prepared by adding 50 ml of 20x SSC and 5 ml of 10% SDS to 445 ml of water. 20x SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

[00125] Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

[00126] Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

[00127] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

Tm = 81.5° C + 16.6 Log[Na+] + 0.41(%G+C) - 0.61(%formamide) - 600/length of duplex in base pairs.

[00128] Washes can typically be carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20° C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

[00129] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

Tm (°C) = 2(number T/A base pairs) + 4(number G/C base pairs)

(Suggs *et al.*, 1981).

[00130] Washes can typically be out as follows:

(1) Twice at room temperature for 15 minutes 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1x SSPE, 0.1% SDS (moderate stringency wash).

[00131] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low:	1 or 2x SSPE, room temperature
Low:	1 or 2x SSPE, 42° C
Moderate:	0.2x or 1x SSPE, 65° C
High:	0.1x SSPE, 65° C.

[00132] Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

[00133] PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (*see* Mullis, U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki *et al.,* 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as *Taq* polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

[00134] Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the

scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

[00135] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target pests and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[00136] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[00137] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus,

"focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[00138] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, or 297 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[00139] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained

using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

[00140] It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, *B.t.* proteins can be used in a truncated (core protein) form (*see, e.g.*, Höfte *et al.* (1989), and Adang *et al.* (1985)). As used herein, the term "protein" can include functionally active truncations.

[00141] In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

[00142] Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93,

94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

[00143] Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, 1990. BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. Gapped BLAST can be used as described in Altschul *et al.*, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. *See* NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, MD, U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

[00144] Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. **Table 5** provides a listing of examples of amino acids belonging to each class.

Table 5

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

[00145] In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

[00146] As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

[00147] Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

[00148] Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

[00149] Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf and pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

[00150] In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this

invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

[00151] Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

[00152] Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

[00153] A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Patent No. 5,135,867.

[00154] Vectors comprising an *AAD-1* polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, *etc*. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids.

Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley *et al.* (1986); and An *et al.* (1985).

[00155] A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

[00156] The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

[00157] In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

[00158] Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate, (*PAT*), glyphosate (*EPSPS*), imazethyapyr (*AHAS*), and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

[00159] Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Patent Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, *see* U.S. Patent Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Patent Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S.

Patent No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Patent No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Patent Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Patent Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. *See* U.S. Patent Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Electroporation technology has also been used to transform plants. *See* WO 87/06614 to Boyce Thompson Institute; U.S. Patent Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Patent No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Patent Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00160] As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

[00161] In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta *et al.* (1980) and EPO 0 120 515.

Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00162] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

[00163] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00164] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

[00165] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00166] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific

promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00167] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00168] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00169] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00170] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Method for Identifying Genes That Impart Resistance to 2,4-D *In Planta*

[00171] As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence

already identified that encodes a protein with the desired characteristics (*i.e.*, α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.,* 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤ 50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00172] PCR and cloning of gene into pET. A single gene (*rdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 28% amino acid identity to *tfdA* from *Ralstonia eutropha*. Percent identity was determined by first translating both the *rdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00173] The strain of *Sphingobium herbicidovorans* containing the *rdpA* gene was obtained from ATCC (American Type Culture Collection strain #700291). The lyophilized strain was revived according to ATCC protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 536. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days.

[00174] A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' TCT AGA AGG AGA TAT ACC ATG CAT GCT GCA CTG TCC CCC CTC TCC CAG CG 3' [(SEQ ID NO:1) (added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' CTC GAG TTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

[00175] Twenty microliter reactions were set up as follows: MasterMix 8 μl, ea. primer 1 μl (50 pmoles/μl), gDNA 2.5 μl, H_2O 7.5 μl. PCR was then carried out under the following

conditions: 94° C 45 sec, 52° C 1.5 minute, 72° C 1.5 minute, for 30 cycles, followed by a final cycle of 72° C 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1kb PCR product was cloned into pCR 2.1 (Invitrogen cat. # K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00176] Ten of the resulting white colonies were picked into 4 ml Luria Broth + 50 μg/ml Kanamycin (LB K), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Promega Wizard Plus SV kit (Promega cat. #A1460) and following the included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward (5' GTA AAA CGA CGG CCA GT 3') (SEQ ID NO:16) and Reverse (5' CAG GAA ACA GCT ATG AC 3') (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein (SEQ ID NO:9) was given a new general designation for internal consistency *AAD-1 (v1)* (AryloxyAlkanoate Dioxygenase).

[00177] Using the restriction enzymes corresponding to the sites added with the primer linkers (Xba 1, Xho 1) *AAD-1 (v1)* was cut out of the pCR2.1 vector and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates. To differentiate between *AAD-1 (v1)*:pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C for 4 hours with agitation.

[00178] Each culture was then spotted onto LB K plates, which were incubated at 37° C overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3203.

Example 2 – Expression and Testing

[00179] 2.1 – HPLC analysis.

[00180] Plasmid pDAB 3203 was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After

transformation, 50 µl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

[00181] The next morning, all colonies from the entire plate were scraped into 100 mls LB in a 500 ml tri-baffled flask and incubated at 37° C/200 rpm for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C/200 rpm. All 100 ml of culture was centrifuged at 4000 rpm for 20 min. The supernatants were then discarded, and the pellets were resuspended in 10 ml of 50 mM MOPS. These were then subjected to three 45-sec rounds of sonication to lyse the cells. Following this, lysates were centrifuged at 15,000 rpm to remove cell debris. The supernatant was pipetted off and stored at 4° C. To check for recombinant expression, a 20 µl aliquot was run on a 4-20% Tris Glycine gel (Invitrogen cat. #EC60255).

[00182] After expression was confirmed, enzyme activity was tested as follows. First, an aliquot of the cell extract was desalted with a PD-10 cartridge (Amersham cat. #17-0435-01). This was then used for subsequent herbicide enzyme reactions.

[00183] For each reaction, the following were combined: 2,4-D (125 µg/ml), [Ascorbate (1 mM), Ferrous ion (50 µM), α-ketoglutarate (1 mM), in 100 mM MOPS], cell extract (100 µl). This reaction was then incubated at room temp for 30 min., after which the reaction was stopped with the addition of 0.1 N HCl until pH was between 2 and 3. Half of the reaction volume (~500 µl) was set aside for bioassay, the remaining volume was organically extracted using Solid Phase Extraction tubes (Fisher cat. #11-131-6), eluting with 400 µl of Acetonitrile + 0.05% TFA.

[00184] The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10µ C18(2) 250 x 4.6mm (Phenomenex cat. #00G-4253-E0), run at 50% ACN + 0.05% TFA: 50% H_2O + 0.05% TFA to 100% ACN + 0.05% TFA over 5 min.

[00185] 2.2 – Plate test bioassays for herbicide degradation.

[00186] Plant bioassays were used to determine if *in vitro* enzymatic herbicide transformation resulted in a concomitant loss in herbicidal activity. Because of the selective nature of the herbicides being tested (*i.e.*, monocot plants controlled by AOPP herbicides and dicot plants controlled by auxinic herbicides), wildtype *Agrostis palustris* var. Pencross and *Arabidopsis thaliana* var. Columbia were used as monocot and dicot test species, respectively. Each species is amenable to germination and growth in small Petri dishes.

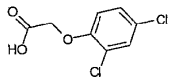
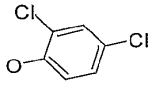
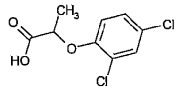
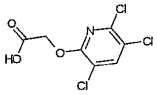
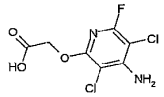
[00187] *Arabidopsis* seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 μL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @ 250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water. Bentgrass seeds were surface sterilized for 20 minutes in a like manner.

[00188] Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4 \cdot 2H_2O$, 10 μM NaCl, 10 nM $CoCl_2 \cdot H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60x15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

[00189] For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day^{-1}, 100 $\mu E/m^2s^1$, 23° C) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

[00190] This bioassay was used to confirm the loss of herbicidal activity as a result of AAD-1 (v1) degradation of the oxyalkanoate side chain from various agronomically relevant herbicides. In several instances, the anticipated phenol product co-eluted with the parent acid on HPLC and the bioassay served as the primary screen for herbicide degradation. **Tables 6 and** 7 represent herbicidal substrates tested.

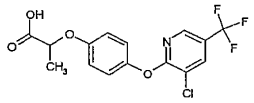
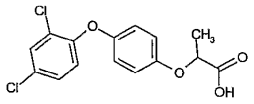
Table 6. *Arabidopsis* plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
2,4-D	22	17	267	16	
DCP	>1000	nd	nd	nd	
Dichlorprop	nd	30	1000	33	
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

* Blank vector represents of cell lysate treatment where E. coli pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

Table 7. Bentgrass plate test bioassay for commercial aryloxyphenoxyalkanoate ACCase-inhibiting substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
Haloxyfop-RS	28	21	520	25	
Diclofop-RS	nd	20	130	7	

* Blank vector represents of cell lysate treatment where E. coli pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

[00191] 2.3 – HPLC results.

[00192] From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see **Figure 1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme for being degraded. Based on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

[00193] Surprisingly, the initial HPLC results showed the presence of a new peak at 6.1 minute, in addition to a reduced 2,4-D peak at 5.5 min. This new peak was not present in the control assay. For an initial identification of the peak at 6.1 minutes, a DCP control was run under our assay conditions and predictably this also eluted at 6.1 minutes. The formation of this product was confirmed using a colorimetric assay to detect phenols (see example 3.1) as well as mass spectrometry. As expected, AAD-1 (v1) carries out a similar reaction as other members of this enzyme class. In the bioassay, these same samples were also shown to have an almost complete loss of 2,4-D herbicidal activity in the *Arabidopsis* plate assay (**Figure 2**). Regardless of the specific conditions of the assay (*i.e.*, longer incubations, more enzyme), only 50-75% of the 2,4-D could be degraded to DCP as measured by HPLC. In fact, longer induction of the BL-21 *E. coli* cells with IPTG only resulted in less active enzyme, even though more total recombinant protein was expressed.

[00194] After demonstrating degradation of 2,4-D, additional substrates were tested with similar ring substitutions (*i.e.*, oxyacetates and oxypropionates). The first compounds tested were the pyridine analogs fluroxypyr and triclopyr, which are pyridinyloxyacetates. No enzyme activity was detected on either of these as substrates. Additional tests on various analogs of these two pyridinyloxyacetates with either the fluorine or the amino groups removed also were not degraded. Interestingly however, adding a fluorine to the 5 position of 2,4-D resulted in an almost total loss of enzyme degradation (see next section for additional results).

[00195] ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (**Figure 3**) and diclofop. These

results were also confirmed by the colorimetric assay, which was also used to test a wider sampling of these compounds.

[00196] 2.4 – Plate test bioassays for herbicide degradation.

[00197] Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurified recombinant AAD-1 (v1) extracts (**Figure 2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR_{50} for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio of 2-3 typically correlated with 50-75% loss of parent herbicide activity (**Table 6**). Often a GR_{50} could not be determined following enzyme treatment; *de facto*, no detectable herbicide activity remained.

[00198] The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (v1) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (**Figure 3** and **Table 7**). These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

Example 3 – *In vitro* assay of AAD-1 (v1) activity via colorimetric phenol detection

[00199] 3.1 – AAD-1 (v1) assay.

[00200] AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides such as haloxyfop and cyhalofop (see **Figure 4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily

detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

[00201] Enzyme assays were performed in a total volume of 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 15 minutes of incubation at 25° C, the reaction was terminated by addition of 10 μl 100 mM sodium EDTA. Color was developed by addition of 15 μl pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH), 1.5 μl 2% 4-aminoantipyrine and 1.5 μl 8% potassium ferricyanide. After 10 to 20 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols. Later assays were made more convenient by consolidating the additions as follows: the reaction was quenched by addition of 30 μl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer and 0.2 % 4-aminoantipyrine, then adding 10 μl 0.8 % potassium ferricyanide.

[00202] 3.2 – Extraction.

[00203] Activity of recombinant AAD-1 (v1) expressed in *Escherichia coli*. *E. coli* cell pellets were resuspended in 0.1 M Tris, pH 7.4 + 1mg/ml lysozyme (5 ml/cells from 250 ml culture; 20 ml/cells from 1 liter) at room temperature. After about 15 minutes with occasional shaking, the suspension was frozen in liquid nitrogen then thawed. DNase was added to 0.02 mg/ml final concentration and $MgCl_2$ to 1 mM. After the extract was no longer viscous, the extract was centrifuged for 15 min. The supernatant was passed over a BioRad 10DG column pre-equilibrated with 20 mM MOPS pH 6.75 and the eluant stored in aliquots at –70° C. Assays were either performed with these unpurified desalted extracts or with purified enzymes.

[00204] A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding *AAD-1 (v1)* was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (v1) extract was compared to that from *E. coli* cells expressing a vector without *AAD-1 (v1)* using 1 mM 2,4-D and is

shown in **Figure 5**. The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

[00205] The activity of this extract was tested on four additional herbicides, (R,S)-dichlorprop, (R,S)-mecoprop, (R,S)-haloxyfop and (R,S)-diclofop in comparison to 2,4-D (all at a final concentration of 0.5 mM) using 4 µl of the *E. coli* extract per assay with a 15 min assay period. **Figure 6A** shows that AAD-1 (v1) cleaved all five herbicides to yield a phenol with the relative activity on the substrates being dichlorprop = mecoprop > diclofop > haloxyfop > 2,4-D. Thus AAD-1 (v1) has activity on graminicidal aryloxyphenoxypropionate herbicides as well as phenoxy auxins.

[00206] The AAD-1 (v1) extract was then tested using racemic (R,S)-haloxyfop, the R enantiomer of haloxyfop and the S-enantiomer of cyhalofop (all at 0.5 mM) as potential substrates to ascertain the likely enantiomeric specificity of AAD-1 (v1). The results are shown in **Figure 6B**. The activity of the enzyme on (R)-haloxyfop was equivalent to that on (R,S)-haloxyfop whereas no activity could be seen on the S-enantiomer of cyhalofop indicating that AAD-1 (v1) has R specificity on AOPPs.

Example 4 – Substrate Specificity of AAD-1 (v1)

[00207] 4.1 – Additional substrates of AAD-1 (v1).

[00208] The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 µg per 160 µl assay and each substrate was tested at 1 mM with an assay time of 15 minutes. **Table 3** shows the A_{510} detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced small amounts of phenol from a variety of phenoxyacetates including 2,4-D. The relative rates on these substrates are better gauged from the assays using the higher amounts (10 µg) of AAD-1 (v1). From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates, X188476 and X398166 (sesone).

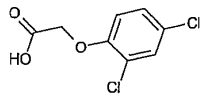
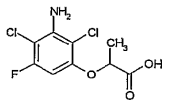
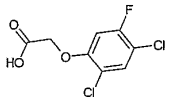
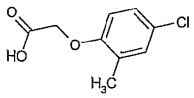
[00209] **Table 4** shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly

increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

[00210] Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness (**Table 8**). X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast to the close analog 3-amino-dichlorprop, which is ~7- fold better than 2,4-D as a substrate (**Table 3**). The 5-F substitution appears to decrease the effectiveness of X11115427 as a substrate for AAD-1 (v1). The rates of product formation from 5-F-phenoxyacetate and MCPA were 32% and 55% that of 2,4-D respectively.

[00211] **Table 8:** Effect of AAD-1 (v1) on three substrates relative to 2,4-D. Substrates were assayed as in Table 6 at 1 mM using a crude recombinant AAD-1 (v1) extract from *E. coli.*

Table 8. Effect of AAD-1 (v1) on three substrates relative to 2,4-D.

Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
195517		2,4-D	0.177	100
11115427		(R,S)-3-amino, 5-F-dichlorprop	0.221	125
124987		5-F, 2,4-D	0.056	32
192711		MCPA	0.097	55

[00212] 4.2—Kinetic characterization.

[00213] The K_m and k_{cat} values of purified AAD-1 (v1) (see Example 10) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μM Na ascorbate; 200 μM Fe^{2+}; 1 mM α-ketoglutarate; 25° C). The dose response curves were fitted using Grafit (Erithacus Software, UK), and the graphs and derived constants are shown in **Figure 7** and **Table 9** respectively. The K_m values for the four substrates were fairly similar (75 – 125 μM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in **Table 3** and **Table 4** as these were performed at high (saturating) substrate concentrations (1 mM).

[00214] **Table 9:** Kinetic Constants for AAD-1 (v1) Substrates. Kinetic constants were derived from the data in Figure 7 using Grafit fitting to the Michaelis-Menten equation.

Table 9. Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (μM) ± SE	V_{max} (μmol min^{-1} mg^{-1} AAD1) ± SE	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
R-dichlorprop	75 ± 10	0.79 ± 0.03	26.1	348
R-quizalofop	125 ± 20	0.57 ± 0.03	18.9	151
R-haloxyfop	120 ± 54	0.34 ± 0.04	11.2	94
2,4-D	96 ± 8	0.57 ± 0.00	2.7	28

[00215] The relative K_m and k_{cat} values for dichlorprop and 2,4-D differ significantly from those published for the R-specific dioxygenase from *Delftia acidovorans* by Westendorf *et al.* (2003) (*Acta Biotechnol.* 23: 3-17). The published k_{cat}/K_m value for 2,4-D is 0.6% that of dichlorprop, whereas in our studies, the k_{cat}/K_m value for 2,4-D is 8% that of dichlorprop. Thus, in this study, AAD-1 (v1) is unexpectedly effective at catalyzing the cleavage of 2,4-D. This increases its potential utility for conferring diverse herbicide tolerance traits in transgenic applications.

[00216] 4.3 – Additional substrates for AAD-1 (v1).

[00217] Three additional substrates were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in **Table 8**. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness. X11115427 was only slightly better (125%) as a substrate than 2,4-D. This is in contrast to 3-aminodichlorprop which is >7 fold better than 2,4-D as a substrate (**Table 3**). Thus, the 5-F substitution has significantly decreased the effectiveness of X11115427 as a substrate for AAD-1 (v1). A similar pattern is seen with 5-F-2,4-D which is only 32% as effective as a substrate relative to 2,4-D. In this assay, MCPA was also less effective as a substrate of AAD-1 (v1) (55% relative to 2,4-D).

Example 5 – Optimization of Sequence for Expression in Plants

[00218] 5.1 – Background.

[00219] To obtain high expression of heterologous genes in plants, it may be preferred to reengineer said genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

[00220] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00221] **Table 10** illustrates how high the G+C content is in maize. For the data in **Table 10**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

Table 10: Compilation of G + C contents of protein coding regions of maize genes

Protein Class.sup.a	Range % G + C	Mean % G + C.sup.b
Metabolic Enzymes (76)	44.4-75.3	59.0 (.+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (.+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (.+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (.+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (.+-.5.2)

.sup.a Number of genes in class given in parentheses.
.sup.b Standard deviations given in parentheses.
.sup.c Combined groups mean ignored in mean calculation

[00222] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A

or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

[00223] In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in **Table 11**. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, *etc.* with the next preferred codon of choice.

Table 11. Preferred amino acid codons for proteins expressed in maize

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

[00224] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00225] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled for the gene sequences for the

particular plant. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Patent No. 5,380,831.

[00226] 5.2 – Rebuild analysis.

[00227] Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native *AAD-1 (v1)* coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5) was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and **Table 12** shows the differences in codon compositions of the native and plant-optimized sequences.

Table 12. Codon composition comparison of native *AAD-1 (v1)* coding region and Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
ALA (A)	GCA	2	9.1	5	22	LEU (L)	CTA	0	0.0	0	0
	GCC	10	45.5	8	35		CTC	5	22.7	7	32
22	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43	22	CTT	0	0.0	8	36
ARG (R)	AGA	0	0.0	7	30		TTA	0	0.0	0	0
	AGG	0	0.0	7	30		TTG	1	4.5	7	32
	CGA	0	0.0	0	0	LYS (K)	AAA	1	14.3	2	29
23	CGC	16	69.6	5	22	7	AAG	6	85.7	5	71
	CGG	4	17.4	0	0	MET (M)	ATG	8	100	8	100
	CGT	3	13.0	4	17	PHE (F)	TTC	10	76.9	9	69
ASN (N)	AAC	8	100.0	4	50	13	TTT	3	23.1	4	31
8	AAT	0	0.0	4	50	PRO (P)	CCA	1	5.9	8	47
ASP (D)	GAC	15	78.9	10	53		CCC	9	52.9	1	6
19	GAT	4	21.1	9	47	17	CCG	7	41.2	0	0
CYS (C)	TGC	3	100.0	2	67		CCT	0	0.0	8	47
3	TGT	0	0.0	1	33	SER (S)	AGC	11	73.3	4	27
END	TAA	0	0.0	0	0		AGT	0	0.0	0	0
1	TAG	1	100.0	0	0	15	TCA	0	0.0	4	27
	TGA	0	0.0	1	100		TCC	2	13.3	3	20
GLN (Q)	CAA	0	0.0	7	54		TCG	2	13.3	0	0
13	CAG	13	100.0	6	46		TCT	0	0.0	4	27
GLU (E)	GAA	8	50.0	5	31	THR (T)	ACA	1	4.3	6	26
16	GAG	8	50.0	11	69		ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29	23	ACG	5	21.7	0	0
	GGC	15	71.4	7	33		ACT	1	4.3	8	35
21	GGG	3	14.3	2	10	TRP (W)	TGG	5	100	5	100
	GGT	3	14.3	6	29	TYR (Y)	TAC	7	70.0	6	60
HIS (H)	CAC	6	60.0	5	50	10	TAT	3	30.0	4	40
10	CAT	4	40.0	5	50	VAL (V)	GTA	2	7.1	0	0
ILE (I)	ATA	0	0.0	3	25		GTC	11	39.3	8	29
12	ATC	12	100.0	5	42	28	GTG	15	53.6	10	36
	ATT	0	0.0	4	33		GTT	0	0.0	10	36
	Totals	148		149			Totals	148		148	

[00228] 5.3 – Completion of binary vectors.

[00229] 5.3.1 – Rebuilt *AAD-1 (v3)*. The plant optimized gene *AAD-1 (v3)* was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-1 (v3)* DNA sequence. The *AAD-1 (v3)* gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB720,

containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-1 (v3)*: Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1 (v3)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, HinD III, Pac I, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

[00230] 5.3.2 – Native *AAD1 (v1)* and modified *AAD-1 (v2)*. The *AAD-1 (v1)* gene (SEQ ID NO:3) was PCR amplified from pDAB3203. During the PCR reaction, alterations were made within the primers to introduce the NcoI and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "rdpA(ncoI)" [CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC] (SEQ ID NO:6) and "3'saci" [GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA] (SEQ ID NO:7) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[00231] The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

[00232] Sequence data identified a clone with the correct sequence. During analysis a superfluous NotI restriction site was identified toward the 3' end of *AAD-1 (v1)*. This site was removed to facilitate cloning into pDAB3038. To remove the additional site a PCR reaction was performed with an internal 5' primer. The NotI site was altered by incorporating a new codon for an amino acid to remove the spurious NotI site. This change would alter the arginine at position 212 to a cysteine. The PCR primers "BstEII/Del NotI" [TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC] (SEQ ID NO:8) and "3' saci" (SEQ ID NO:7) were used.

[00233] A PCR reaction was completed using the Fail Safe PCR System (Epicenter) and the resulting fragment was cloned into the pCR 2.1 TOPO TA cloning kit (Invitrogen). Confirmation of the correct PCR product was completed by DNA sequencing, and the "fixed" gene was given the designation *AAD-1 (v2)* (SEQ ID NO:4).

[00234] A sequencing reaction using the M13 Reverse primer (SEQ ID NO:17) and the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents indicated that the correct PCR fragment had been isolated. This construct was digested with the BstEII and SacI enzymes. The resulting fragment was cloned into the pCR2.1 *AAD-1 (v2)* construct (pCR2.1 Delta NotI) and confirmed via restriction enzyme digestion.

[00235] The modified *AAD-1 (v2)* gene was then cloned into pDAB726 as a NcoI/SacI DNA fragment. The resulting construct (pDAB708) was verified with restriction digests. This construct was then cloned into the binary pDAB3038 as a NotI - NotI fragment. The final resulting construct was given the designation pDAB766, containing the [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1 (v2)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] and was restriction digested for verification of the correct orientation. The completed construct was then used for transformation into *Agrobacterium*.

[00236] 5.3.3 – Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is presented herein as SEQ ID NO:20. A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is presented herein as SEQ ID NO:21.

[00237] A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in **Table 13**. Columns D and H of **Table 13** present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the

protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in **Table 13.** Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

Weighted % of C1 =1/(%C1 + %C2 + %C3 + *etc.*) x %C1 x 100

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of **Table 13** (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of **Table 13**. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

Table 13. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A	B	C	D	E	F	G	H
Amino Acid	**Codon**	**Weighted %**	**Soybean %**	**Amino Acid**	**Codon**	**Weighted %**	**Soybean %**
ALA (A)	GCA	33.1	30.3	**LEU (L)**	CTA	**DNU**	**9.1**
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	**DNU***	**8.5**		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9		TTA	**DNU**	**9.8**
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	**DNU**	**8.2**	**LYS (K)**	AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
	CGG	**DNU**	**6.0**	**MET (M)**	ATG	100.0	100
	CGT	16.9	14.5	**PHE (F)**	TTC	49.2	49.2
ASN (N)	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0	**PRO (P)**	CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1		CCC	20.9	19.2
	GAT	61.9	61.9		CCG	**DNU**	**8.3**
CYS (C)	TGC	50.0	50.0		CCT	39.3	36.0
	TGT	50.0	50.0	**SER (S)**	AGC	16.0	15.1
END	TAA	**DNU**	40.7		AGT	18.2	17.1
	TAG	**DNU**	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5		TCG	**DNU**	**6.1**
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	**THR (T)**	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9		ACG	**DNU**	**8.3**
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4	**TRP (W)**	TGG	100.0	100
	GGT	30.4	30.4	**TYR (Y)**	TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8		TAT	51.8	51.8
	CAT	55.2	55.2	**VAL (V)**	GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

***DNU** = Do Not Use

[00238] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall

weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21.

[00239] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is given as bases 1-1575 of SEQ ID NO:22. Synthesis of a DNA fragment comprising SEQ ID NO:22 was performed by a commercial supplier (PicoScript, Houston TX).

[00240] 5.3.4 - Cloning of additional binary constructs. The completion of pDAB3295 and pDAB3757 incorporated the use of the GateWay Cloning Technology (Invitrogen, cat #11791-043 and cat #12535-019). The GateWay Technology uses lambda phage-based site-specific recombination to insert a gene cassette into a vector. For more information refer to *Gateway Technology: A universal technology to clone DNA sequence for functional analysis and expression in multiple systems*, © 1999-2003, Invitrogen Corp., 1600 Faraday Ave., Carlsbad, CA 92008 (printed - 2003). All other constructs created for transformation into appropriate plant species were built using similar procedures as above and other standard molecular cloning methods (Maniatis *et al.*, 1982). **Table 14** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

[00241] The *sacB* gene was added to the binary vector pDAB3289 as a bacterial negative selection marker to reduce the persistence of *Agrobacterium* associated with transformed plant tissue. SacB is a levan-sucrase enzyme produced by *Bacillus* spp. and is toxic to most Gram negative bacteria when grown in the presence of sucrose (Gay *et al.*, 1983). The *sacB* gene was recovered on a *Hind* III fragment from plasmid pRE112 (Edwards, *et al.*, 1998) and cloned into the unique *Hind* III site in pDAB3289.

Table 14. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Trans-formed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	AAD1 v3	CsVMV	Agro binary
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	HptII	AtUbi3	Agro binary
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Aerosol beam
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3403		Cn, R	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	Same as GOI		Whiskers / Gun
3404		Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	pat	OsAct1	Whiskers
3415	1283	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers
3602	1421	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Spectinomycin	-	AHAS v3	OsAct1	Agro Superbinary
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi11	Agro binary
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	-	-	Erythromycin	-	pat	CsVMV	Agro binary

*A = *Arabidopsis*
T = Tobacco
S = Soybean
Ct = Cotton
R = Rice
Cn = Corn
Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region

(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region

ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
HptII = hygromycin phosphotransferase

Example 6 – Transformation into *Arabidopsis* and Selection

[00242] 6.1 – *Arabidopsis thaliana* growth conditions.

[00243] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00244] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00245] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2 – *Agrobacterium* transformation.

[00246] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

[00247] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 μl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00248] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant

agitation for 4 hours. After incubation, the culture was plated on YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00249] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28°C for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP + erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed Agrobacterium cells, suspended in 10 µl of water, at 100° C for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wisconsin) per manufacturer's instructions at 0.5x concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C for 3 minutes, 2) 94° C for 45 seconds, 3) 55° C for 30 seconds, 4) 72° C for 1 minute, for 29 cycles then 1 cycle of 72° C for 10 minutes. The reaction was maintained at 4° C after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

[00250] 6.3 – *Arabidopsis* transformation.

[00251] *Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C with constant agitation. The cells were then pelleted at approx. 8700x g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: 1/2x Murashige and Skoog

salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044μM benzylamino purine (10 μl/liter of 1 mg/ml stock in DMSO) and 300 μl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

[00252] 6.4 – Selection of transformed plants.

[00253] Freshly harvested T_1 seed (transformed with native [*AAD-1 (v2)*] or plant optimized [*AAD-1 (v3)*] gene) was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00254] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00255] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)* (plant optimized gene) or *AAD-1 (v2)* (native microbial gene) to provide phenoxy auxin herbicide resistance.

[00256] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR_{99} for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200g ae/ha).

[00257] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of ~7-8.

[00258] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded *in planta*.

[00259] Although *Arabidopsis*, being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, *AAD-1 (v3)*-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above Injury ratings were taken 7 and 14 days after treatment. Likewise, T_1 individuals were treated with the pyridyloxyacetate auxin herbicide, fluroxypyr.

[00260] 6.5 – Results of selection of transformed plants.

[00261] The first *Arabidopsis* transformations were conducted using *AAD-1 (v3)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493

glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (*see* **Table 15**). A small lot of *AAD-1 (v2)* (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

Table 15: Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Codon bias	Total seed sown and screened	Number of resistant T_1	Selection rate	Selection rate range	% of selected plants expressing PAT[3]
Glufosinate[1]	*AAD-1 (v2)*	n	84,000	278	0.33%	0.33%	nd[4]
Glufosinate[1]	*AAD-1 (v3)*	p	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D[2]	*AAD-1(v3)*	p	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate[1]	*AAD-2 (v1)*	n	1,301,500	228	0.018%	0.007 to 0.021%	100%
Glufosinate[1]	*AAD-2(v2)*	p	200,000	224	0.11%	0.11%	nd[4]

[1]Glufosinate selection scheme: 280g ai/ha glufosinate applied 5-6 + 10 DAP
[2]2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP
[3]PAT protein expression determined by PAT ELISA strips
[4]nd, not determined
[5]codon bias, n-native microbial gene, p=plant optimized

[00262] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** compares the response of native *AAD-1 (v2)* and plant optimized *AAD-1 (v3)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Both genes imparted resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)* or *AAD-1 (v3)* versus the wildtype or *PAT/Cry1F* transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence *AAD-1 (v3)* versus the native sequence *AAD-1 (v2)* (see **Table 16**). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T_1 individuals expressing AAD-1 (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6x field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

[00263] **Table 16.** *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 16. *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence.

	% Injury			% Injury	
Native AAD-1 (v2) gene Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control-buffer	20	6	7	25.3	34.7
50 g ae/ha 2,4-D	55	16	9	14.8	22.7
200 g ae/ha 2,4-D	45	11	24	34.1	39.3
800 g ae/ha 2,4-D	11	32	37	52.5	34.2
	% Injury			% Injury	
Native AAD-2 gene Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control-buffer	4	1	1	25.0	21.7
50 g ae/ha 2,4-D	1	2	11	68.2	30.2
200 g ae/ha 2,4-D	0	3	11	82.7	28.8
800 g ae/ha 2,4-D	0	0	14	99.8	0.8
	% Injury			% Injury	
Rebuilt AAD-1 (v3) gene Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control-buffer	9	0	0	0.0	0.0
50 g ae/ha 2,4-D	10	1	5	24.3	35.9
200 g ae/ha 2,4-D	11	4	1	14.0	25.9
800 g ae/ha 2,4-D	11	4	1	14.7	26.1
	% Injury			% Injury	
Wildtype Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.0	0.0
200 g ae/ha 2,4-D	0	0	15	95.1	0.5
800 g ae/ha 2,4-D	0	0	15	100.0	0.0
	% Injury			% Injury	
PAT/Cry1F (transformed control) Averages	<20%	20-40%	>40%	Ave	Std Dev
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.7	4.2
200 g ae/ha 2,4-D	0	0	15	97.2	1.7
800 g ae/ha 2,4-D	0	0	15	100.0	0.0

[00264] **Table 17** shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400 – 1,600 g ae/ha (**Table 18**). Normal field use rate for haloxyfop (a grass-specific

herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some *AAD1 (v3)* transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first *in planta* data that *AAD-1 (v3)* will provide AOPP resistance. No resistance was observed with fluroxypyr (a pyridyloxyacetate auxin) in transformed *Arabidopsis*, consistent with *in vitro* work using heterologously expressed enzyme.

[00265] **Table 17.** T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 17. T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence.

AAD-1 v3	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	7	1	0	5.0	7.6	0-20
50 g ae/ha RS-dichlorprop	7	1	0	3.1	8.8	0-25
200 g ae/ha RS-dichlorprop	4	1	3	40.0	50.1	0-100
800 g ae/ha RS-dichlorprop	0	5	3	51.9	40.0	20-100
PAT/Cry1F	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	0	6	2	38.1	25.3	20-95
50 g ae/ha RS-dichlorprop	0	0	8	80.0	25.3	50-100
200 g ae/ha RS-dichlorprop	0	0	8	98.3	2.2	95-100
800 g ae/ha RS-dichlorprop	0	0	8	100.0	0.0	100
Wildtype	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	3	0	0	13.3	2.9	10-15
50 g ae/ha RS-dichlorprop	0	0	3	53.3	5.8	50-60
200 g ae/ha RS-dichlorprop	0	0	3	95.0	5.0	90-100
800 g ae/ha RS-dichlorprop	0	0	3	100.0	0.0	100

[00266] **Table 18.** T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T1 is an independent transformation event, one can expect significant variation of individual T1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 18. T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide.

AAD-1 v3	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20
800 g ae/ha haloxyfop	1	1	2	46.3	42.7	0-85
1600 g ae/ha haloxyfop	1	0	3	65.0	47.3	0-100
PAT/Cry1F	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	10.0	0.0	10
400 g ae/ha haloxyfop	0	4	0	27.5	5.0	20-30
800 g ae/ha haloxyfop	0	0	4	78.8	6.3	70-85
1600 g ae/ha haloxyfop	0	0	4	47.5	43.5	80-100
Wildtype	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	0	3	0	20.0	0.0	20
800 g ae/ha haloxyfop	0	0	3	73.3	10.4	70-85
1600 g ae/ha haloxyfop	0	0	3	93.3	11.5	80-100

[00267] 6.6 – *AAD-1 (v3)* as a selectable marker.

[00268] The ability to use *AAD-1 (v3)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T_1 seed

transformed with *PAT* and *AAD1 (v3)* (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active *PAT* gene.

[00269] Fifty-three out of 70,000 seed planted were identified as resistant to 2,4-D. ELISA was used to screen a subset of 44-individuals from this population for PAT protein expression. Ninety six percent of the individuals were positive indicating the presence of the co-transformed gene, *PAT*. The low number of negative ELISA results (4%) is in line with a 3% error rate in populations of glufosinate-resistant plants (**Table 15**). The efficiency of selection appears to be somewhat less with 2,4-D (0.08%) vs. glufosinate (0.12%); however, the range of selection rates across all experiments would indicate both selection agents are equally good for selecting *Arabidopsis* transformed with *AAD-1 (v3)* or *PAT* genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

[00270] 6.7 – Heritability.

[00271] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 60% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00272] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show *AAD1 (v3)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

[00273] 6.8 –Additional herbicide resistance attributable to AAD-1 in *Arabidopsis*.

[00274] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified *in vitro* plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4 homozygous plants id = PAAD1.315.064) were sterilized by agitating for 10 min in a 50% bleach solution. These seeds were then rinsed four times with sterile water to remove the bleach.

[00275] Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration of DMSO never exceeded 1% (v/v). After thorough mixing, a 6 mL aliquot of the warm media containing the appropriate concentration of compound was added to each well of a 6-well, flat bottom, polystyrene tissue culture tray (Falcon 353046, Becton Dickson and Company, Franklin Lakes, NJ). After the media solidified, approximately 20 to 30 *Arabidopsis* seeds were applied on top of the solidified media and the remaining 2 mL of media was poured over the seeds. The plates were lightly agitated to disperse the seeds, covered and allowed to cool until the media had completely solidified. The plates were incubated for 7 days at 25°C under continual fluorescent lighting (75 $\mu E\ m^{-2}\ s^{-1}$). Nutrient Media Composition was as described in Example 2.2 and in Somerville and Orgen (1982).

[00276] Assessment of growth reduction. The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in **Table 19**. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All

commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported *in planta*. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of *AAD-1*-transformed commercial crops or experimental plant species.

Table 19. *In vitro* plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

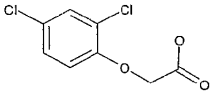
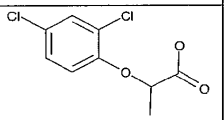
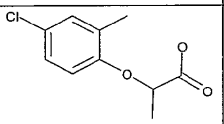
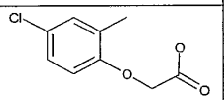
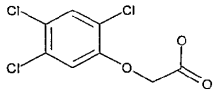
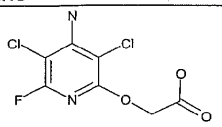
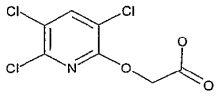
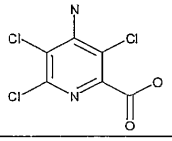
Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Phenoxy auxins							
2,4-D		0.2	10	< 0.01	0.04	50	> 4
dichlorprop		2	5	0.01	1.5	2.5	150
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		1.5	10	<0.01	<0.01	6.67	NA

Table 19. *In vitro* plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Pyridine auxins							
Fluroxypyr		2	1	0.2	0.2	0.5	1
Triclopyr		0.2	0.04	0.02	0.02	0.2	1
Picloram		1	0.5	0.3	0.15	0.5	0.5

[00277] 6.9 – Foliar applications herbicide resistance in AAD-1 *Arabidopsis* .

[00278] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates described in Example 6.8. T_4 generation *Arabidopsis* seed, homozygous for *AAD-1 (v3)* (line AAD1.01.315.076) was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of phenoxy auxin herbicides: 12.5-1600 g ae/ha 2,4-D dimethylamine salt (DMA) (Riverside Chemicals), 12.5-1600 g ae/ha mecoprop (AH Marks), 50-3200 g ae/ha R-dichlorprop (AH Marks), 8.75-1120 g ae/ha 2,4,5-T (technical grade); pyridyloxyacetates herbicides 50-3200 g ae/ha triclopyr (Dow AgroSciences) and 50-3200 g ae/ha fluroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-1 activity, 2,4-dichlorophenol (DCP, Sigma) (at 50-3200 g ae/ha, technical grade). All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment and are averaged over two experiments.

[00279] These results (see **Table 20**) confirm that *AAD-1 (v3)* in *Arabidopsis* provides robust resistance to the phenoxyacetic auxins, phenoxypropionic auxins, but have not shown significant cross resistance to the pyridyloxyacetic auxins tested and corroborates the *in vitro* enzyme and whole plate substrate specificity data. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on wildtype or transgenic *Arabidopsis*.

Table 20. Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Phenoxypropionic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**PatCry1f - Control**
50 g ae/ha R-Dichlorprop	3	31
200 g ae/ha R-Dichlorprop	3	73
800 g ae/ha R-Dichlorprop	3	89
3200 g ae/ha R-Dichlorprop	3	95
12.5 g ae/ha Mecoprop	3	0
25 g ae/ha Mecoprop	0	2
50 g ae/ha Mecoprop	0	17
100 g ae/ha Mecoprop	0	33
200 g ae/ha Mecoprop	3	62
400 g ae/ha Mecoprop	0	78
800 g ae/ha Mecoprop	0	93
1600 g ae/ha Mecoprop	0	100
Phenoxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Cry1f - Control**
12.5 g ae/ha 2,4-D DMA	0	67
25 g ae/ha 2,4-D DMA	0	78
50 g ae/ha 2,4-D DMA	0	93
100 g ae/ha 2,4-D DMA	0	100
200 g ae/ha 2,4-D DMA	0	100
400 g ae/ha 2,4-D DMA	0	100
800 g ae/ha 2,4-D DMA	0	100
1600 g ae/ha 2,4-D DMA	0	100
8.75 g ae/ha 2,4,5-T	0	0
17.5 g ae/ha 2,4,5-T	3	20
35 g ae/ha 2,4,5-T	0	43
70 g ae/ha 2,4,5-T	3	85
140 g ae/ha 2,4,5-T	0	95
280 g ae/ha 2,4,5-T	0	98
560 g ae/ha 2,4,5-T	17	100
1120 g ae/ha 2,4,5-T	3	100

Table 20. Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

	Pyridyloxyacetic auxins	
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Cry1f - Control**
50 g ae/ha Triclopyr	31	36
200 g ae/ha Triclopyr	58	65
800 g ae/ha Triclopyr	74	84
3200 g ae/ha Triclopyr	97	95
50 g ae/ha Fluroxypyr	48	76
200 g ae/ha Fluroxypyr	75	85
800 g ae/ha Fluroxypyr	88	85
3200 g ae/ha Fluroxypyr	95	95
	Inactive DCP metabolite	
50 g ae/ha 2,4-DCP	0	0
200 g ae/ha 2,4-DCP	0	0
800 g ae/ha 2,4-DCP	0	0
3200 g ae/ha 2,4-DCP	0	0

[00280] 6.10 – Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

[00281] An experiment was designed to examine if the level of AAD-1 (v3) expression in *Arabidopsis* varies at different growth stages. A high-tolerance, homozygous, *AAD-1 (v3)* T_4 line (id=PAAD1.01.345.163) was grown in greenhouse. Half of the plants were treated with 800 g ae /ha of 2,4-D (as previously described) while the other half were not treated. Two leaves, the 3rd leaf from the top and the 5th leaf from the bottom, were harvested from 5 plants, both treated and untreated, and analyzed by ELISA and Western Blotting (as described in Example 11) experiments at 4, 10, 14, 20 and 25 DAT. **Figures 8A** and **8B** showed that there was statistically no difference in AAD-1 (v3) expression between young and old leaves. In addition, the herbicide 2,4-D had little impact on the expression level of AAD-1 (v3) protein. The protein levels accumulated in older plants with some significant protein degradation at the later time points.

[00282] In a separate experiment, four different homozygous T_4 lines of *Arabidopsis* displaying different tolerance level to the herbicide 2,4-D were sprayed with various levels (0, 200, 800 and 3200 g/ha) of 2,4-D and their herbicide injury and AAD-1 (v3) expression were examined. Four days after the herbicide treatment, little injury was observed in three of the four lines, even at the highest dose tested (**Figure 9A**). These plants also expressed high

level of AAD-1 (v3), from 0.1 to 0.25% of (**Figure 9B**). On the contrary, the low tolerance line expressed less than 0.1% of AAD-1 (v3) in TSP and suffered observable injury. More importantly, they recovered from the injury on 14 DAT (**Figure 9A**), indicating that the low level of AAD-1 (v3) expression was able to protect the plants from the serious herbicide damage. All control plants suffered serious injury and died 14 DAT at doses 800 g ae/ha 2,4-D and above.

[00283] 6.11 – Molecular analysis of *AAD-1 (v3) Arabidopsis*.

[00284] Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number and/or Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-1 (v3)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-1 (v3)*. Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00285] For Southern analysis, a total of 1 µg of DNA was subjected to an overnight digest of Nsi I for pDAB721 to obtain integration data. The samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of *PAT*. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then

subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at -80° C.

[00286] Results showed all 2,4-D resistant plants assayed contained *PAT* (and thus by inference, *AAD-1 (v3)*). Copy number analysis showed total inserts ranged from 1 to >10 copies. This correlates, too, with the AAD-1 (V3) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance (>>200 fold) to all commercially available phenoxyacetic and phenoxypropionic acids.

[00287] 6.12 – *Arabidopsis* transformed with molecular stack of *AAD-1 (v3)* and glyphosate resistance gene.

[00288] T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid (*AAD-1 (v3)* + *EPSPS*) coding for a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-1 (v3)* as the selectable marker as described in example 6.6, except the 2,4-D rate used was 75 g ae/ha. Twenty-four T_1 individually transformed events were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-1 (v3)* + *PAT* T_5 homozygous lines (pDAB721-transformed), and *PAT* + *Cry1F* homozygous line (transformed control). Only pDAB3230 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in 200 mM Hepes buffer (pH 7.5). All treatments were replicated 4 or 5 times. Plants were evaluated 7 and 14 days after treatment. I_{50} values were calculated and show >14 fold level of tolerance imparted by *EPSPS* molecularly stacked with *AAD-1 (v3)* (*see* **Figure 10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate + 2,4-D treatments applied in tankmix as described in Example 21 and shown for *AAD-1 (v3)*-transformed corn in Example 8.

Example 7 – WHISKERS-Mediated Transformation Into Maize, and Use of *AAD-1 (v3)* as a Selectable Marker

[00289] 7.1 – Cloning of *AAD-1 (v3)*.

[00290] The *AAD-1 (v3)* fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and SacI and the 5175 bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 (OsAct1/PAT/ZmLip) were digested with NotI. The 3442 bp band from pDAB3403 and the 11017 bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5α, and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404, which contains ZmUbi1/po-aad1/ZmPer5::OsAct1/PAT/ZmLip.

[00291] 7.2 – Callus/suspension initiation.

[00292] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00293] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00294] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS

Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L *myo*-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

[00295] 7.3 – Cryopreservation and thawing of suspensions.

[00296] Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C/minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

[00297] For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 mL H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until

a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

[00298] 7.4 – Dose response of non-transformed tissue to haloxyfop acid.

[00299] Non-transformed donor Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 200 mg of callused suspension tissue was transferred per treatment to selection media in 60x20 mm plates containing 30, 100 or 300 nM R-haloxyfop acid. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos (from Herbiace commercial formulation, Meiji Seika, Japan), GN6 (1H), was also included for comparison. Callus was removed after 2 weeks, weighed, and transferred to fresh media of the same concentration for another 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. Results are shown in **Figure 11**.

[00300] Two separate dose response studies of callus tissue to the phenol degradation products of haloxyfop and cyhalofop, respectively, were also completed to confirm that this end product would not be deleterious to callus growth. Data from a cyhalofop phenol dose response (see **Figure 12)** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol. Data from a haloxyfop phenol dose response showed that even at 300 nM haloxyfop phenol, growth was equal to or greater than the control lacking haloxyfop phenol (data not shown).

[00301] 7.5 – WHISKERS-mediated transformation using bialaphos selection.

[00302] Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L *myo*-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker in the dark for 30-35 minutes, and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume of GN6 S/M

liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00303] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00304] Either 3 or 6 mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

[00305] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (1H) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 1.0 mg/L bialaphos; 2.5 g/L Gelrite, pH 5.8) or GN6D (1H) medium (same as GN6 (1H) except with 8.0 mg/L dicamba and 0.8 mg/L 2,4-D).

[00306] Plates were placed in boxes and cultured as before for an additional week. Two weeks post-transformation, the tissue was embedded by scraping either ½ the cells on the plate or else all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C) containing 1 mg/L bialaphos. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (1H) or GN6D (1H) medium. This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C in dark boxes.

[00307] Putatively transformed isolates were typically first visible 5-8 weeks post-transformation. Any potential isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained

growth was evident after approximately 2 weeks, an event was deemed to be resistant and was submitted for molecular analysis.

[00308] Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (1H), containing 1 mg/L bialaphos, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week then higher light (40 $\mu Em^{-2}s^{-1}$) for another week before being transferred to regeneration medium, 36 (1H), which was identical to 28 (1H) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

[00309] 7.6 – WHISKERS-mediated transformation using haloxyfop selection.

[00310] DNA delivery parameters for direct selection on "fops" were identical to the bialaphos selection procedure except that 85 µg of pDAB3403 and 85 µg of construct containing a GFP (Green Fluorescent Protein) reporter gene were co-transformed together, and only 3 mL of suspension was filtered onto GN6 medium following the 2-hour recovery.

[00311] After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60x20-mm plates of GN6 medium containing 2 mg/L 2,4-D plus 50, 100, or 200 nM R-haloxyfop acid. Plates were placed in boxes and cultured for one additional week. After one week, the tissue was embedded by scraping all cells from the plate into 3.0 mL of melted GN6 agarose medium containing the same concentration of selection agent as in the previous transfer. All steps afterward were identical to the PAT selection/regeneration protocol except that 100 nM R-haloxyfop acid was included in the regeneration media instead of 1 mg/L bialaphos.

[00312] 7.7 – Results. Multiple experiments testing various levels of haloxyfop and cyhalofop were initiated, and 47 isolates were recovered from direct selection. A subset of the callus events were submitted for screening using PCR and Western analyses. Following these expression data, 21 lead events were submitted for Southern analysis. Results using NcoI, a unique cutter, to obtain integration data following probing with *AAD-1 (v3)*, unequivocally demonstrate stable integration of *AAD-1 (v3)* following Whiskers-mediated transformation coupled with "fop" selection.

[00313] 7.8 – Quantitative demonstration of *in vitro* tolerance from AAD-1 (v3) expressing callus events from bialaphos selection

[00314] Ninety-seven callus isolates recovered from bialaphos selection were submitted for PAT copy number via Invader analysis and *AAD-1 (v3)* PTU analysis via PCR (see Example 7.10). AAD-1 (v3) protein expression using Western blot/Sandwich ELISA (Example 11) was completed on a subset of the events. A summary is described in **Table 21** below. At least 15 T_0 plants were regenerated from each of these events and sent for spray testing and seed production.

Table 21.

Event	PAT Copy #	PCR for *AAD-1 (v3)* PTU	Callus Western
3404-001	2	+	+
3404-006	2	+	+
3404-013	3	+	+
3404-017	1	+	+
3404-020	3	+	nd
3404-022	2	+	+
3404-025	2	+	+
3404-027	3	+	+
3404-031	1	+	+
3404-033	2	+	nd
3404-036	3	+	+
3404-044	3	+	+
3404-050	3	+	+
3404-053	3	+	+
3404-074	2	+	+
3404-082	2	+	+

[00315] A smaller subset of these events was assessed in dose-response studies in comparison to a non-transformed control. A range of concentrations of haloxyfop (from Gallant Super formulation), up to 400 nM, were tested. Dose response data for one event, 3404-006, was generated using the general methods of Example 7.4, and is shown in **Figure 13**. This demonstrates that event 3404-006 showed no significant reduction in callus growth at haloxyfop concentrations up to 400 nM whereas non-transgenic corn callus tissue growth was inhibited at this rate. These data have not been normalized to account for inherent growth differences that are not related to the expression of the transgene.

[00316] 7.9 –WHISKERS-mediated transformation using imazethapyr selection.

[00317] The ZmUbi1/*AAD-1 (v3)*/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the *AAD-1 (v3)* and *AHAS* Whiskers transformation vector pDAB3415, which is also referred to as pDAS1283) Once completed, this construct was transformed into maize via silicon carbide whiskers-mediated transformation as described in Example 7.4 except 2 mL of cells were filtered, followed by a 7-day recovery on GN6 medium followed by selection on media containing 3 μM imazethapyr from Pursuit® DG herbicide. Following Invader analysis, 36 events were identified that contained the AAD-1 (v3) and the AHAS genes.

[00318] Fifty-three corn calli events from these transformations were tested in both ELISA and Western Blotting experiments for their AAD-1 (v3) expression – a subset of the data is shown below. For the events listed in **Table 22,** the expression levels of those detected positive ranged from 90 to over 1000 ppm of total soluble proteins.

Table 22.		
AAD-1 Event ID Number	**Expression Level (ppm)**	**Western Blot**
1283[1]-001	2	-
1283[1]-002	206	+++
1283[1]-003	1	-
1283[1]-004	90	+++
1283[1]-005	1	-
1283[1]-006	105	+++
1283[1]-007	212	++
1283[1]-008	114	±
1283[1]-009	305	+
1283[1]-010	2	-
1283[1]-011	4	-
1283[1]-012	200	+++
1283[1]-013	134	+++
1283[1]-014	4	-
1283[1]-015	194	+++
1283[1]-016	4	-
1283[1]-017	196	+++
1283[1]-018	3	-
1283[1]-019	178	+
1283[1]-020	260	++
1283[1]-021	144	+++
1283[1]-022	140	+++
1283[1]-023	191	+++
1283[1]-024	392	++
1283[1]-025	368	++
1283[1]-026	14	-
1283[1]-027	1006	++
Neg Control	3	-
Neg Control	3	-
Standard (0.5 µg/mL)		++
Standard (5 µg/mL)		++++

[00319] 7.10– Molecular Analysis: Maize Materials and Methods.

[00320] 7.10.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00321] 7.10.2 – Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis was used to identify the estimated copy of the unknown events.

[00322] 7.10.3 – Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-1 (v3)* PTU were (Forward – ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:25) and (Reverse – CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:26). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR *AAD-1 (v3)* were (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse – CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00323] 7.10.4 – Southern blot analysis. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µg of DNA was subjected to an overnight digestion of Afl II and also EcoRV for pDAB3404, NcoI for pDAB3403, and SpeI for pDAB1421 to obtain integration data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC was then set up to

obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using the above coding region PCR using plasmid pDAB3404. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at -80° C.

Example 8 – *In vivo* tolerance and field tolerance data generated from *PAT*-selected (pDAB3404) *AAD-1 (v3)* events

[00324] 8.1 - Tolerance of T_0 corn plants to AOPP herbicides.

[00325] If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (*i.e.*, plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light: 8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure® II (DuPont), Clincher* (Dow AgroSciences), or Gallant Super* (Dow AgroSciences) for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v Agridex crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1X

lethal dose (~1/8 X field dose) up to 8X field doses (64 X lethal dose). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

[00326] AOPP's are generally very potent corn-killing herbicides. Three to four leaf Hi-II corn grown from seed is effectively killed (>95% injury) with 8.8, 62.5, and 4.4 g ae/ha of haloxyfop, cyhalofop, and quizalofop, respectively. Each *AAD-1 (v3)*-transformed line tested survived a minimally lethal dose of each AOPP herbicide tested. In fact, most lines tested survived with no visible injury (14 DAT) even when treated with an 8X field dose (64X lethal dose) of quizalofop. Several individual clones from events "017" and "038," however, did incur significant injury at elevated rates. This could be a function of lower gene expression due to how or where the gene was inserted.

[00327] The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (T_0 stage). Significantly, this tolerance was shown for all three AOPP herbicides and likely will extend to all AOPP herbicides as previously shown for AAD-1 *in vitro*.

[00328] **Figure 14** shows the responses of several *AAD-1 (v3)*–transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied 1 week prior.

[00329] **Table 23** shows data for the responses of selected *AAD-1 (v3)*-transformed T_0 corn events to three AOPP herbicides applied postemergence.

Table 23. Selected AAD-1 (v3)-transformed T0 corn events response to three AOPP herbicides applied postemergence.

Construct	Event	Clone	Haloxyfop* g ae/ha	Cyhalofop** g ae/ha	Quizalofop*** g ae/ha	% Injury 14 DAT
3404	001	016	8.8			0
3404	001	018		62.5		0
3404	001	017			8.8	0
3404	001	019			8.8	30
3404	001	020			35	0
3404	017	018			35	0
3404	017	019			35	0
3404	017	020			70	0
3404	017	021			70	0
3404	017	022			140	0
3404	017	023			140	30
3404	017	024			280	30
3404	017	025			280	20
3404	022	019	8.8			0
3404	022	020	17.5			0
3404	022	016	17.5			0
3404	022	024		62.5		0
3404	022	018		125		0
3404	022	021			8.8	0
3404	022	017			17.5	0
3404	022	022			35	0
3404	022	023			70	0
3404	033	012			35	0
3404	033	013			70	0
3404	033	014			70	0
3404	033	015			140	0
3404	033	016			280	0
3404	038	016	8.8			0
3404	038	018		62.5		0
3404	038	017			8.8	0
3404	038	019			35	70
3404	038	020			35	80
3404	038	021			70	80
3404	038	022			70	80
			(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 g ae/ha)	(lethal dose = 4.4 g ae/ha)	

*Gallant super[#] + 1% COC (v/v)
**Clincher [#] + 1% COC (v/v)
***Assure II + 1% COC (v/v)
[#]Trademark of Dow AgroSciences, LLC

8.2 – Field tolerance of pDAB3404 T_1 corn plants to quizalofop 2,4-D, and glufosinate herbicides.

[00330] Two field trials were established at field stations in Hawaii and Indiana. Corn seed from inbred T_1 plants were utilized to evaluate sixteen AAD1 event lines for tolerance against quizalofop and 2,4-D. Three non-transformed hybrids were included for comparison purposes. The hybrid Hi-II x 5XH571 is of the same parentage as the *AAD-1 (v3)* event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

[00331] The experimental design was a split-plot with four replications. The main plot was herbicide treatment and the sub-plot was *AAD-1 (v3)* event or comparison hybrid. Plots were one row by 3.7 meters with approximately twenty-five seeds planted in each row. For AAD1 events, seeds from a different lineage within the event were planted in each replicate.

[00332] Glufosinate at 560 g ai/ha was applied to *AAD-1 (v3)* plots at the V2 stage to eliminate non-transformed plants. Experimental treatments included commercial formulations of quizalofop applied at 70 and 140 g ae/ha, 2,4-D (dimethylamine salt) at 560 and 1120 g ae/ha, and an untreated control. Treatments were applied using backpack broadcast boom equipment delivering 187 L/ha carrier volume at 130-200 kpa pressure. Quizalofop treatments were applied at the V3-V4 corn stage and 2,4-D treatments were applied at the V5-V6 stage.

[00333] Quizalofop treated plots were visually assessed for crop injury at one and three weeks after application (WAA) using a 0-100% scale, where 0 equals no injury and 100 equals complete death. 2,4-D treated plots were visually assessed for plant leaning at 2 days after application (DAA) using 0-100% scale where 0 equals no leaning from any plant and 100 equals all plants prone. Additionally, 2,4-D plots were visually assessed at 3-4 WAA for brace root deformation using a 0-10 scale.

[00334] 8.2.1 – Results.

[00335] *AAD-1 (v3)* event response to the highest rates tested of quizalofop and 2,4-D are shown in **Table 24**. These rates represent approximately twice the normal commercial use rates. Non-transformed hybrids were severely injured (80-100%) by quizalofop at 70 g ae/ha including the sethoxydim resistant line, although it displayed slightly better tolerance than the other two hybrids. All *AAD-1 (v3)* events except one lineage of event 3404.001 displayed excellent tolerance to quizalofop at 70 g ae/ha. No visible symptoms were observed on the *AAD-1 (v3)* events except with the events noted above.

Table 24.

Treatment (rate) =	Quizalofop (140 g ae/ha)		2,4-D amine (1120 g ae/ha)		2,4-D amine (1120 g ae/ha)		Analysis Results			Resistance Segregation Ratios following Liberty Spray		
Evaluation =	% Injury 3 WAA (0-100 scale)		% Leaning 2 DAA (0-100 scale)		Braceroot Deformation 3-4 WAA (0-10 scale)		AAD-1 copy number leaf (Southern analysis)	AAD-1 Leaf Western Blot T_0	AAD-1 Leaf ELISA T_0	T_1 Population Average		T_2 Population Average
Event or Hybrid	IN	HI	IN	HI	IN	HI				IN	HI	HI
3404.001	25	25	0	5	1	2	3	+++	+++	40%	47%	X
3404.006	0	0	0	0	0	0	1	++	+++	33%	26%	X
3404.013	0	0	0	0	0	0	1	+	+++	63%	58%	X
3404.017	0	0	1	0	0	0	2	+/-	++	48%	47%	X
3404.020	0	0	0	0	0	0	2	++	+++	50%	51%	X
3404.022	0	0	1	0	0	0	1	+	++	51%	57%	76%*
3404.025	0	0	0	0	0	0	2	+++	++++	55%	59%	X
3404.027	0	0	3	0	0	0	5	+	++	51%	50%	X
3404.031	0	0	1	0	0	0	1	++	++++	47%	43%	61%*
3404.033	0	0	0	0	0	0	2 or 3	+	++	52%	49%	X
3404.036	0	0	0	0	0	0	4	++	+++	52%	48%	X
3404.044	0	0	1	0	0	1	1	+	+/-	50%	48%	X
3404.050	0	0	0	1	0	1	2	nd	nd	38%	28%	X
3404.053	0	0	1	0	0	0	2	+++	+++	48%	56%	X
3404.074	0	0	0	0	0	0	1	++	+++	53%	52%	73%*
3404.082	0	0	0	0	0	1	3	nd	nd	38%	36%	X
DK493	100	100	20	23	8	9						
HI-II X 5XH571	100	100	13	34	7	9						
CROPLAN 585SR	80	96	11	33	9	9						

* Fits single locus dominant trait segregation as determined by chi square analysis (P>0.05)

[00336] 2,4-D at the 1120 g ae/ha rate caused significant levels (11-33%) of epinastic leaning in the non-transformed hybrids, a normal response when applied beyond the V4 growth stage. Little or no leaning was observed with all *AAD-1 (v3)* events except one lineage of 3404.001 (Indiana location only) where moderate levels (5-13%) of leaning occurred.

[00337] Brace roots of non-transformed hybrids were severely deformed (rating of 9 on a 0-10 scale) by 2,4-D at the 1120 g ae/ha rate. Again, this is a normal response to 2,4-D applied beyond the V4 growth stage. As with the leaning response, little or no brace root injury was observed with all *AAD-1 (v3)* events except one lineage of 3404.001.

[00338] Similar trends occurred with lower tested rates of quizalofop and 2,4-D although at reduced but still significant response levels in the non-transformed hybrids (data not shown).

[00339] These results indicate that most *AAD-1 (v3)* transformed event lines displayed a high level of resistance to quizalofop and 2,4-D at rates that were lethal or caused severe epinastic malformations to non-transformed corn hybrids. *See also* **Figure 16.**

[00340] 8.2.2- Expected Mendelian segregation ratios on three T_2 events. Plants from individual lineages of each event were randomly self-pollinated in the field. T_2 seed were hand harvested at physiological maturity. Based on single gene copy number (see **Table 24** above) and overall performance in T_1 generation (segregation, herbicide tolerance, and vigor), three events (022, 031, and 074) were chosen for further evaluation in the field. Breeding rows of each event were planted using a precision cone planter each consisting of 2500-3000 seeds. At the V2 growth stage, all *AAD-1 (v3)* lines were sprayed with 140 g ae/ha quizalofop (Assure® II) using a backpack sprayer as previously described. This rate rapidly killed all "null" (untransformed) segregants. Each event had a segregation ratio consistent with Mendelian inheritance of a single locus, dominant gene (3 resistant:1 sensitive, or 75% survival) (see **Table 24**). Homozygotes and hemizygotes from event 74 were identified by zygosity testing (refer to *AAD-1 (v3)* Invader assay description for corn). Hemizygous plants were removed and homozygous *AAD-1 (v3)* plants were crossed with BE1146 corn inbred introgressed and homozygous for glyphosate resistance trait, NK603, creating a homogeneous F_1 hybrid seed that is hemizygous for glyphosate resistance, *AAD-1 (v3)*, and glufosinate resistance.

[00341] 8.3 – Stacking of *AAD-1 (v3)* and *PAT* with glyphosate resistance genes in corn.

[00342] Homozygous T_2 *AAD-1 (v3)/PAT* corn plants were crossed with glyphosate resistant corn plants producing F_1 seed containing *AAD-1 (v3)*, *PAT*, and glyphosate resistance genes as described in the previous example.

[00343] F_1 seeds were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium (Sun Gro Horticulture). The pots were initially subirrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were subirrigated with deionized water.

[00344] Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. The plants were sprayed with rates of 2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200

mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

[00345] At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Results of the study at 14 DAT can be seen in **Table 25.**

Table 25.

	Field Rate	% Injury at 14 DAT	
		Hi II X 5XH751	RR/PAT/AAD1
		Ave	Ave
Untreated control	-	0	0
840 g ae/ha glyphosate	1X	98	0
1680 g ae/ha glyphosate	2X	100	0
3360 g ae/ha glyphosate	4X	100	0
560 g ae/ha 2,4-D DMA	1X	10	0
1120 g ae/ha 2,4-D DMA	2X	14	0
2240 g ae/ha 2,4-D DMA	4X	29	0
470 g ae/ha glufosinate	1X	80	0
940 g ae/ha glufosinate	2X	90	3
1880 g ae/ha glufosinate	4X	96	15
840 g ae/ha glyphosate + 560 g ae/ha 2,4-D DMA	1X + 1X	96	1
1680 g ae/ha glyphosate +1120 g ae/ha 2,4-D DMA	2X + 2X	100	2
3360 g ae/ha glyphosate + 2240 g ae/ha 2,4-D DMA	4X + 4X	100	1
470 g ae/ha glufosinate + 560 g ae/ha 2,4-D DMA	1X + 1X	89	5
940 g ae/ha glufosinate + 1120 g ae/ha 2,4-D DMA	2X + 2X	91	10
1880 g ae/ha glufosinate + 2240 g ae/ha 2,4-D DMA	4X + 4X	97	13
840 g ae/ha glyphosate + 470 g ae/ha glufosinate	1X + 1X	90	5
1680 g ae/ha glyphosate + 940 g ae/ha glufosinate	2X + 2X	98	15
3360 g ae/ha glyphosate + 1880 g ae/ha glufosinate	4X + 4X	100	15

[00346] This study demonstrated that the *AAD-1 (v3)* gene in corn can be stacked with a glyphosate resistance gene and a glufosinate resistance gene to provide robust field-level tolerance to 2,4-D, glyphosate, and glufosinate alone or in tank mix combinations.

[00347] 8.3.1 – Resistance of *AAD-1 (v3)* corn using a tank mix of 2,4-D DMA and quizalofop. T_2BC_1 seeds of hemizygous event number 3404-025.001R/R001 Bulked.001.S058 were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium. The pots were initially sub-irrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were sub-irrigated with de-ionized water.

[00348] Plants were allowed to grow until V1 stage in the greenhouse. At this point the plants were selected with 560 g ae/ha Assure® II with the addition of 1% Agridex crop oil concentrate in 200 mM Hepes buffer with the research track sprayer set at 187 L/ha. Plants were allowed 4 days to show symptoms of the selection. All plants were uninjured. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 18-in spray height. All applications were formulated in 200 mM Hepes buffer (pH 7.5) with the addition of 1% v/v Agridex.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Plants from this particular lineage had 0% injury at 14 DAT for all tank mixed combinations, while the wild-type had 100% injury. These results indicate AAD-1 (v3) not only provides robust field level resistance to 2,4-D and quizalofop individually, but also to exaggerated rates of multiple combinations of the two chemistries. One could logically expect to implement novel weed control measures with combinations of phenoxy auxins and AOPP graminicides in corn (or other crops transformed with AAD-1) not previously enabled by a single gene HTC.

[00349] 8.3.2 - Tolerance of (pDAB3403) T_0 corn plants to quizalofop herbicide. A target of approximately eight T_0 plant clones from each of 17 events were regenerated and transferred to the greenhouse for preliminary tolerance screening with postemergence-applied discriminating rate of quizalofop herbicide applied by track sprayer at 35 g ae/ha (1X field rate, 4X lethal dose) to 3-leaf, greenhouse-adapted T_0 corn plants using track sprayer conditions previously described. Plants were rated as Resistant or Sensitive 7 days after treatment. Control, non-transgenic corn was included with each spray application. Two events, Event 014 and 047, had two or more T_0 clones sensitive to 35 g ae/ha quizalofop, indicating an unexpected level of sensitivity for this event. The 15 other events showed stable integration, protein expression, and the ability to tolerate a 4X lethal dose of quizalofop at the whole plant level.

[00350] 8.3.3 Expression of AAD-1 (v3) with respect to quizalofop tolerance.

[00351] Three different T2 lineages from 3404 transformations that were pre-screened with Liberty® (as described previously) to remove nulls were chosen to compare their tolerance to quizalofop with respect to their AAD-1 (v3) expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT (see **Figure 15.**). The highest tolerance line, event

3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1X and higher field rates. This data concludes that corn expressing AAD-1 (v3) can be protected from quizalofop injury at the highest level tested (2,240 g/ha), which is 16 times the 1X field dose of 35 g/ha. In addition, the expression level was consistent throughout the period of the experiment.

Example 9 - *Agrobacterium*-Mediated Transformation of Maize with *AAD-1 (v3)*

[00352] 9.1 - Plant material.

[00353] Seeds of a "High II" (*i.e.*, Parent A and B) F_1 cross (Armstrong *et al.*, 1991) are planted directly into 5 gallon-pots containing 95:5 Metro-Mix® 360: Mineral soil. The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps.

[00354] 9.2 - Tissue source.

[00355] For obtaining immature Hi-II (F2) embryos, controlled sib-pollinations were performed. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. Immature embryos were isolated as described in Example 7.2.

[00356] 9.3 - Preparation of a superbinary vector.

[00357] Construction of an *Agrobacterium* construct, pDAB2272, containing the *AAD-1 (v3)* gene in combination with the AHAS selectable marker gene was accomplished by isolating the 3443 base pair NotI fragment from pDAB3404 containing ZmUbi1 v2/ *AAD-1 (v3)*/ZmPer5 v2 and inserting it into the NotI site of pDAB8549. The resulting plasmid contains the ZmUbi1 v2/ *AAD-1 (v3)*/ ZmPer5 v2 and the OsAct1 v2/AHAS v3/ZmLip v1 cassettes flanked by non-identical MAR regions in the direct orientation. This was subsequently transformed into LBA4404/pSB1 to create the superbinary vector, which was named pDAB3602 but was also referred to as pDAS1421.

[00358] 9.4 - Bacterial supply.

[00359] All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Patent No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The

bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~ $1x10^9$ cfu/ml) and 100 μM actetosyringone added to the solution.

[00360] 9.5 - Infection and cocultivation.

[00361] The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod liquid medium. Each tube, containing ~100 embryos, is vortexed for 3-5 sec. The medium is removed and replaced with fresh liquid medium and the vortex is repeated. The liquid medium is again removed and this time replaced with an *Agrobacterium* solution at the Klett 200 concentration. The *Agrobacterium* and embryo mixture is vortexed for 30 sec. Following a 5 minute incubation at room temperature, the embryos were transferred to LS-As Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L $AgNO_3$,1, 100 μM actetosyringone, 3.0 g/L Gelrite, pH 5.8) for a 5-day co-cultivation at 25° C.

[00362] 9.6 - Dose response using immature embryos.

[00363] Dose response studies were initiated using immature embryos treated with *Agrobacterium* strain LBA4404 lacking a plasmid as described previously. Once treated, embryos were allowed to co-cultivate for 5 days at 25° C and were then transferred to selection media containing various levels of R-haloxyfop or R-cyhalofop. Embryos were also transferred to media containing 1 mg/L bialaphos and 100 nM imazethapyr as negative controls. Embryos were scored for % embryogenic callus formation after 2 weeks, and then again after 4 weeks. Embryos were tested on R-haloxyfop levels up to 30 nM; however, insufficient reduction of callus formation was seen at the highest levels, so higher concentrations (50 – 100 nM) were used during transformation experiments. Data from embryos grown on cyhalofop-containing media is shown in **Figure 17**.

9.7 – Selection.

[00364] After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then

moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the *AHAS* gene is used, based on U.S. Patent No. 5,731,180. Tissue was transferred at biweekly intervals on the same medium until embryogenic colonies were obtained. These colonies were maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies were bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

[00365] 9.8. – Regeneration and seed production.

[00366] For regeneration, the cultures are transferred to 28 "induction" medium and 36 "regeneration" medium as described previously containing either 100 nM R-haloxyfop or 1.5 μM cyhalofop for differentiation of plantlets. When plantlets were established, they were transferred to SHGA tubes to allow for further growth and development of the shoot and roots as described previously. Controlled pollinations for seed production were conducted as described previously.

[00367] 9.9 - Event recovery and analysis particulars; whole plant screening of T_0 corn lineages containing *AAD-1 (v3)* and *AHAS* (pDAS1421).

[00368] Seventy-two *Agrobacterium*-transformed events were selected on various levels of R-haloxyfop acid and R-cyhalofop acid *in vitro*. Twenty-two callus samples were analyzed by Southern blot analysis for stable integration of *AAD-1 (v3)* into the genome as described previously. Ten single copy events, as indicated in **Table 26,** were chosen to be regenerated.

Table 26.

Corn event	*In vitro* Selection agent	Southern Copy # (callus)	Western Blot (AAD-1) T_0 Callus	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop	
	(nM)	AAD-1		Resistant	Sensitive
1421[21]-016	50 Haloxyfop	1	+	8	0
1421[22]-020	100 Haloxyfop	1	++	8	0
1421[22]-022	100 Haloxyfop	1	+	8	0
1421[22]-023	100 Haloxyfop	1	++	8	0
1421[3]-036	100 Haloxyfop	1	++	8	0
1421[4]-031	300 Cyhalofop	1	++	9	0
1421[4]-032	300 Cyhalofop	1	++	8	0
1421[4]-033	300 Cyhalofop	1	++	12	0
1421[4]-034	300 Cyhalofop	1	++	8	0
1421[4]-035	300 Cyhalofop	1	++	6	0
Events with more than 1 copy were not taken to the greenhouse					

[00370] A minimum six regenerated clonal lineages per event were moved to soil in the greenhouse and screened using a track sprayer as previously described to apply 35 g ae/ha quizalofop when 2-4 new, normal leaves had emerged (see section 8.3.3). Presence of AAD-1 protein on a Western blot correlated perfectly with herbicide resistance in the T_0 generation regardless of which AOPP herbicide was used for selection. There is no negative impact of the second HTC gene (*AHAS*) on the function of the *AAD-1 (v3)*.

Example 10 – Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

[00371] All operations during purification were carried out at 4° C. Frozen or fresh *E. coli* cells from approximately 1 L culture, grown and induced as in Example 3, were re-suspended in 200 ml of extraction buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 2 ml of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The soluble extract was obtained by centrifugation in a GSA rotor (Sorvall) at 12,000 rpm (24,000g) for 20 minutes. The supernatant was then loaded onto a Mono Q ion exchange column (Pharmacia HR 10/10) equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, and the column was washed with same buffer for 10 CV (80 ml). The protein was eluted with 80 ml of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 ml fractions were collected. The fractions containing AAD-1 (v1) were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM DTT, pH 8.0 at a flow rate of 1 ml/min. Purification procedures were analyzed by SDS-PAGE, and protein concentration was determined by Bradford assay using bovine serum albumin as standard.

Example 11 – Recombinant AAD1 Purification and Antibody Production

[00372] Plasmid pDAB3203 containing the *AAD-1 (v1)* gene was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 µL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were

scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000x g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

[00373] All AAD-1 (v1) purifications were conducted at 4° C as discussed in Example 10, unless otherwise stated. The cell lysate was loaded onto a Mono Q ion exchange column (Pharmacia Cat. #HR 10/10) equilibrated with 20 mM Tris-HCl (pH 8.0) 1 mM EDTA, followed by 80 mL of washing with the same buffer. The proteins were eluted with 80 mL of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 mL fractions were collected. The fractions containing AAD-1 were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 1 mM DTT. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

[00374] Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

[00375] 11.1 – Extracting AAD-1 (v3) from plant leaves.

[00376] Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 µL plant extraction buffer (PBS containing 0.1 % Triton X-100 and 10 mM DTT). The tubes were shaken for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000x g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

[00377] 11.2 – Bradford assay.

[00378] Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five micro-liter of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For standards, concentrations were ranged from 2000 to 15.6 μg/mL. The protein assay concentrate was first diluted 5 fold in PBS and 250 μL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

[00379] 11.3 – Enzyme linked immuno-sorbent assay (ELISA).

[00380] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-1 antibody (0.5 μg/mL) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 μL standard AAD-1 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve, purified AAD-1 concentrations ranged from 100 to 1.6 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-1 antibody-HRP conjugate (0.25 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 μL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-1 in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[00381] For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

[00382] 11.4 – Western blotting analysis.

[00383] Plant extracts or AAD-1 standards (5 and 0.5 μg/mL) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane

using standard protocol. After blocking in 4% skim milk in PBS, AAD-1 protein was detected by anti-AAD1 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham cat. #RPN 21058).

Example 12 – Tobacco Transformation

[00384] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721, *AAD-1 (v3) + PAT*), grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

[00385] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[00386] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-1 (v3)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00387] The DNA samples were diluted to 9 ng/µl, then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl2 (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the florometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events (**Table 27**).

Table 27. Tobacco T0 events transformed with pDAB721 (*AAD-1(v3) + PAT*).

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (µg AAD-1 / ml plant extract)	Relative tolerance T0 spray with 2,4-D *
721(1)1	1	+	0.9	Medium
721(2)1	nd	nd	0.6	Medium
721(2)2	5	+	0.3	Low
721(2)3	3	+	2.6	Medium
721(2)5	5	+	4.1	Variable
721(2)6	3	+	0.5	Variable
721(2)8	5	+	0.3	High
721(2)11	3	+	n/a	High
721(2)12	3	+	4.1	Medium
721(2)13	2	+	0.5	Medium
721(2)14	5	+	0.2	High
721(2)16	4	+	3.2	Medium
721(2)17	3	+	nd	High
721(2)18	5	+	nd	High
721(2)19	>10	+	nd	Low
721(2)20	5	+	nd	Medium
721(2)21	4	+	nd	High
721(2)22	7	+	nd	Medium
721(2)23	>10	+	nd	Variable
721(3)003	3	+	nd	Variable
721(3)008	2	+	nd	High
721(3)012	1	+	nd	High
721(3)4	2	+	0.5	High
721(3)5	9	+	3.3	High
721(3)6	4	+	7.1	Variable
721(3)9	2	+	1	Low
721(3)10	3	+	0.6	High
721(3)11	7	+	6	Low
721(3)13	4	+	0.1	High
721(3)014	2	+	0.1	Medium

nd = not done

Legend:	Relative tolerance*	Injury at 3200 g ae/ha 2,4-D (14DAT)
	Low	>50% injury
	Medium	20-50% injury
	High	<20% injury
	Variable	inconsistent

[00388] Copy number estimations were confirmed by Southern Analysis on several events. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µgs of DNA was subjected to an overnight digest of NsiI and also HindIII for pDAB721 to obtain integration data. After the overnight digestion an aliquot of ~100 ngs

was run on a 1% gel to ensure complete digestion. After this assurance the samples were processed using same protocol as in Example 6 section 11.

[00389] All events were also assayed for the presence of the *AAD-1 (v3)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the Coding Region PCR AAD-1 were (RdpAcodF ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:27) and (RdpAcodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 30 PCR positive events were regenerated and moved to the greenhouse.

[00390] A representative plant from each of 19 events was assayed for AAD-1 (v3) expression by ELISA methods previously described. All events assayed showed detectable levels of AAD-1 (v3) (**Table 27**). Protein expression varied across events.

[00391] T_0 plants from each of the 30 events were challenged with a wide range of 2,4-D sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 50, 200, 800, or 3200 g ae/ha to representative clones from each event mixed in deionized water. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 800 g ae/ha or less. Some events were uninjured at this rate (equivalent to 1.5X field rate). All events suffered some level temporary auxinic damage 3 DAT when treated with 3200 g ae/ha. Some leaf burning also occurred at this high rate due to the acidity of the spray solution. Future trials at high 2,4-D rates were buffered. Response of T_0 plants treated with 3200 g ae/ha 2,4-D (~6X field rate) was used to discern relative tolerance of each event into "low" (>50% injury 14 DAT), "medium" (20-50% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (**Table 27**).

[00392] Verification of high 2,4-D tolerance.

[00393] Two to four T_0 individuals surviving high rates of 2,4-D were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. Two *AAD-1 (v3)* tobacco lines (event 721(2)-013.010 and 721(3)-008.005) were chosen from the T_0 generation. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4), followed by selective removal of untransformed nulls in this segregating population with 280 g ai/ha glufosinate (PAT selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided medium and high levels of robustness to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187L/ha. The plants were sprayed from a range of 70-4480 g ae/ha 2,4-D dimethylamine salt (DMA), R-Dichlorprop, and a 50/50 mix of the two herbicides. All applications were formulated in 200mM Hepes buffer (pH 7.5). Each treatment was replicated 4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity. (**Table 28**). No injury was observed at rates below 1X field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by *AAD-1 (v3)*, even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

Table 28. Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.

Herbicide	KY160 – Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
560 g ae/ha 2,4-D DMA	75	5	0
1120 g ae/ha 2,4-D DMA	80	5	2
2240 g ae/ha 2,4-D DMA	90	5	0
4480 g ae/ha 2,4-D DMA	95	5	5
560 g ae/ha R-dichlorprop	70	5	0
1120 g ae/ha R-dichlorprop	75	5	0
2240 g ae/ha R-dichlorprop	88	5	0
4480 g ae/ha R-dichlorprop	95	10	5
560 g ae/ha 2,4-D DMA/R-dichlorprop	80	5	5
1120 g ae/ha 2,4-D DMA/R-dichlorprop	80	10	10
2240 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15
4480 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15

[00394] A 100 plant progeny test was also conducted on each of the two *AAD-1 (v3)* lines (events 721(2)-013.010 and 721(3)-008.005). The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Both event '013' and '008' segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-1 is heritable as a robust phenoxy auxin resistance gene in multiple species.

[00395] Field level tolerance will be demonstrated by planting T_1 or T_2 seed in the greenhouse, selectively removing the null plants by Liberty selection as previously described, and rearing individual seedlings in 72-well transplant flats (Hummert International) in Metro 360 media according to growing conditions indicated above. Individual plants will be transplanted into the field plots using an industrial vegetable planter. Drip or overhead irrigation will be used to keep plants growing vigorously. Once plants reach 6-12 inches in height, tobacco plants will be sprayed with a broad range of phenoxy auxins and rated as shown above. Environmental stresses are more significant under field conditions; however, based on previous experience with *AAD-1 (v3)*-transformed corn, robust translation of resistance from the greenhouse to the field is expected.

Example 13 – Soybean Transformation

[00396] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00397] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-1 (v3)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-1 (v3)*.

[00398] 13.1 – Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00399] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly

dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00400] 13.1.1 – *Agrobacterium* preparation. The plasmid, pDAB721, contains the *AAD-1 (v3)* gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the *PAT* gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

[00401] *Agrobacterium* cultures harboring pDAB721 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

[00402] 13.1.2 – Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to

germinate for 5 days at 24° C, with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.*, 1988).

[00403] Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 μM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

[00404] Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin , 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

[00405] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from10 mg to 100 mg/L. The elongation medium consisted of B5

medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

[00406] The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

[00407] 13.1.3 – Progeny evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1 (v3)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of *AAD-1 (v3)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

[00408] 13.2 – Transformation Method 2: "No-shake" *Agrobacterium* mediated transformation of non-regenerable soybean suspension cells.

[00409] The DAS Soybean cell suspensions were cultured on a 3d cycle with 10 ml of settled suspension volume transferred to 115 ml of fresh liquid medium. Settled cell volume was determined by allowing the cell suspension to settle for 2 min in the 125-mL flask after vigorous swirling and then drawing cells from the bottom of the flask with a wide bore 10 ml pipette. The flasks were then transferred to orbital shakers at 140 rpm.

[00410] Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100x25 sterile Petri plate. EHA105 *Agrobacterium* suspension at a density of 1.2 OD^{650} in a 100 μL volume was added and mixed well. The *Agrobacterium* and suspension cell mixture was swirled well and the plate was transferred to dark growth chamber where the temperature was maintained at 25° C.

[00411] 13.2.1 – Selection of soybean suspension cells and isolation of transformed colonies. After 4 days of co-cultivation the plate was swirled again to mix the suspension well and a 1.5 ml aliquot was transferred to the selection medium and spread on the gel medium on a 100x15 ml Petri-plate. The selection medium consisted of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin, 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose and the medium was amended with glufosinate ammonium at 5 mg/L level. After a 10 min drying in the hood the plates were incubated for 4 weeks in dark at 28° C. Colonies appeared in selection and a total of 11 colonies were transferred to fresh medium from 3 different experiments and maintained for 3-4 months. All the 11 resistant colonies produced calli that were growing on the 5 mg/L glufosinate containing selection medium. The non-transformed suspension cells were sensitive when plated on to 0.5 mg/l glufosinate ammonium medium. However, the transformed colonies were resistant to 5x concentration of glufosinate ammonium and were maintained for up to 4 months.

[00412] Callus events were sampled for analyses when they reached 2 to 3 cm in diameter. At least two of colony isolates, one each from two different experiments, were analyzed for AAD1 protein expression. The ELISA and Western analysis carried out on these two isolates showed positive expression of AAD1 proteins. Both ELISA (**Table 29**) and Western Blotting (**Figure 18**) analysis on two separate soybean calli transformed with *AAD-1 (v3)* gene indicated that the callus cells are expressing AAD-1 (v3) protein. The sandwich ELISA detected 0.0318% and 0.0102% total soluble protein of AAD-1 (v3) in two different callus tissue samples. Due to the sensitivity and cross reactivity of the antiserum, multiple bands were observed in the western blot. However, AAD-1 (v3) specific band was observed in both callus samples but not in the wild type (negative) tissue. Coding region PCR analyses showed the expected size products of the AAD1 and the PAT coding regions in these colonies indicating that they were transformed.

Table 29. PCR and ELISA data for transgenic soybean events.

Event	AAD-1 Coding region PCR	PAT Coding region PCR	T SP (µg/mL)	AAD-1 (ng/mL)	% Expression
1-1	+	+	1995.13	633.89	0.0318%
2-1	+	+	2018.91	205.92	0.0102%
Negative Control	-	-	2074.63	-1.22	-0.0001%

[00413] 13.3 – Transformation Method 3: aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00414] Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Patent No. 6,809,232 (Held *et al.*).

[00415] Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144 and 96E692, were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3295 using linearized DNA at a concentration of approximately 0.2 µg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos. With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing 1/2 B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

[00416] Variations of media referred to in **Table 30** were tested, *e.g.*, B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and 1/2 B5G which contained all ingredients of B5G medium at half strength.

Table 30. Growth Media for Soybean			
	Ingredients in 1 Liter		
	B1-30	B3	B5G
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8g	8g	
Gelrite			2g
pH	5.8	5.8	5.8

Example 14 -- *AAD-1 (v3)* Enablement in Cotton

[00417] 14.1 – Cotton transformation protocol.

[00418] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (**Table 31**) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00419] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (**Table 31**) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (**Table 31**) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (**Table 31**). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00420] *Agrobacterium* preparation. Inoculate 35ml of Y media (**Table 31**) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (**Table 31**) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

[00421] After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. Callus is then transferred to CG-media (**Table 31**), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (**Table 31**) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

[00422] Larger, well-developed embryos are isolated and transferred to DK media (**Table 31**) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

Table 31. Media for Cotton Transformation

Ingredients in 1 liter	**G**	**M liquid**	**M**	**MG**	**CG**	**D**	**DK**	**Y**
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1mM)		1ml	1 ml	1 ml	4.6 ml		0.5ml	
2,4-D (1mM)		1ml	1 ml	1 ml				
agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

[00423] 14.2 – Experiment specifics.

[00424] For this experiment 500 cotyledon segments were treated with pDAB721. Of the 500 segments treated, 475 had callus isolated while on selection (95% transformation frequency). The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader were initiated to determine the insertion patterns and to be sure the gene was present at the callus stage, then callus lines that were embryogenic were sent for Western analysis.

[00425] 14.3 – Callus analysis results.

[00426] The object of the analysis is to eliminate any lines that do not have the complete PTU, show no expression or that have high copy number, so those lines are not regenerated. Of the 475 pDAB721 transformed callus lines, 306 were sent for PCR analysis and Invader assay (**Table 32**). Very few lines were PCR negative. The Invader results are not complete at this time, because some samples had low DNA amounts when extracted, and these have been resubmitted. However, the current Invader data shows a few of the lines submitted have high copy number (copy number of >2) (**Table 32**). Due to the large number of lines that passed analysis, it was necessary to decrease the number of embryogenic callus lines being maintained due to the volume. Ninety lines have been sent for Western analysis, and eight of those were negative. The western analysis showed high expression from the majority of the lines (**Table 32**). Eighty-two embryogenic callus lines are being maintained for plant regeneration based on analysis results (and results pending).

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western	Line	Copy #	PTU	Western
1	2	pos	***	43	1	pos	*****
2	1	pos	***	44	low DNA	pos	*****
3	1	pos	***	45	1	pos	*****
4	2	pos	***	46	2	pos	*****
5	1	pos	***	47	1	pos	***
6	1	pos	neg	48	1	pos	***
7	1	pos	****	49	3	faint pos	neg
8	1	pos	*****	50	1	pos	****
9	2	pos	*	51	4	pos	neg
10	1	pos	*	52	2	pos	****
11	1	pos	*****	53	1	pos	***
12	1	pos	*	54	1	pos	****

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western
13	1	pos	*
14	1	pos	**
15	1	pos	****
16	1	pos	*****
17	1	pos	*
18	1	pos	***
19	1	pos	**
20	1	pos	*****
21	2	pos	*****
22	1	pos	*****
23	1	pos	*****
24	4	pos	* or neg
25	1	pos	****
26	1	pos	****
27	low DNA	pos	*****
28	low DNA	pos	**
29	low DNA	pos	*****
30	17	pos	*
31	low DNA	pos	*****
32	low DNA	pos	*****
33	low DNA	pos	****
34	low DNA	faint pos	*****
35	low DNA	pos	****
36	low DNA	pos	neg
37	low DNA	pos	****
38	low DNA	neg	*****
39	1	pos	****
40	low DNA	pos	*
41	low DNA	pos	*
42	low DNA	pos	**
standard	AAD1	5ug/ml	*****
standard	AAD1	0.5ug/ml	**

Line	Copy #	PTU	Western
55	1	pos	neg
56	5	pos	*
57	2	faint pos	neg
58	8	pos	****
59	2	pos	****
60	5	pos	*****
61	1	pos	**
62	1	pos	**
63	1	pos	***
64	1	pos	***
65	3	pos	****
66	5	pos	*
67	6	pos	*
68	Low DNA	pos	neg
69	Low DNA	pos	****
70	low DNA	pos	**
71	low DNA	faint pos	**
72	low DNA	pos	****
73	low DNA	neg	***
74	low DNA	faint pos	***
75	Low DNA	pos	***
76	Low DNA	pos	neg
77	low DNA	faint pos	****
78	low DNA	pos	****
79	1	pos	**
80	low DNA	pos	***
81	low DNA	pos	***
82	low DNA	pos	***
83	low DNA	neg	****
84	low DNA	pos	***
85	low DNA	pos	**
86	low DNA	pos	*

[00427] 14.4 – Plant regeneration.

[00428] Two *AAD-1 (v3)* cotton lines have produced plants according to the above protocol that have been sent to the greenhouse. To demonstrate the *AAD-1 (v3)* gene provides resistance to 2,4-D in cotton, both the *AAD-1 (v3)* cotton plant and wild-type cotton plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed at 560 g ae/ha 2,4-DMA formulated in 200 mM Hepes buffer (pH 7.5). The plants were evaluated at 3, 7, and 14 days after treatment. Plants were assigned injury ratings with respect to stunting,

chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Three days after treatment (DAT) the wild-type plant began showing epinasy and received a rating of 15%; in contrast, the *AAD-1 (v3)* plant showed 0% injury. By 7 DAT epinasy continued on the wild-type and the new growth shoots began turning brown. It received a rating of 50% at this time. At 14 DAT the *AAD-1 (v3)* plant was still uninjured, whereas the wild-type was severely stunted, and the new growth areas were brown and shriveled. Thus, the wild-type received a rating of 90% at 14 DAT.

[00429] This study demonstrates that the *AAD-1 (v3)* gene in cotton provides substantial tolerance to 2,4-D up to at least 560 g ae/ha.

Example 15 – *Agrobacterium* Transformation of Other Crops

[00430] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g.,* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.*, 1997.

[00431] The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *AAD-1 (v3)*, for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa*, *perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans

(*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, and other families), and Clover (*Leguminosae*). Such plants, with *AAD-1 (v3)* genes, for example, are included in the subject invention.

Example 16 – Stacking *AAD-1 (v3)* with AHAS Herbicide Resistance Gene

[00432] Stacking *AAD-1 (v3)* with an AHAS herbicide resistance gene is described in Example 7.9.

Example 17 – Further Evidence of Surprising Results: *AAD-1 vs. AAD-2*

[00433] 17.1 – *AAD-2 (v1)* initial cloning.

[00434] Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00435] The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site

(RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

[00436] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H_2O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00437] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA GT 3'] (SEQ ID NO:16) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[00438] 17.2 – Completion of *AAD-2 (v1)* binary vector.

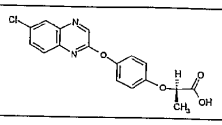
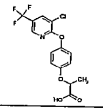
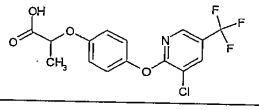
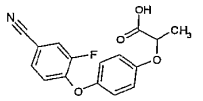
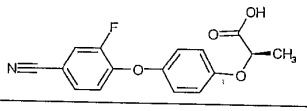
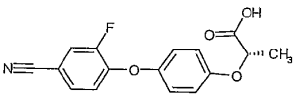
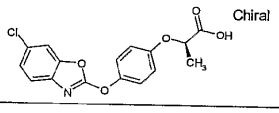
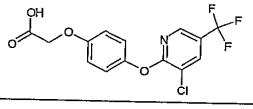
[00439] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a

clone with the correct sequence (pDAB716). The AflIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI – NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: *AAD-2 (v1)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HinDIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

[00440] 17.3 – Comparison of substrate specificities of AAD-2 (v1) and AAD-1 (v1)

[00441] The activity of an extract from *E. coli* expressing AAD-2 (v1) (pDAB3202) prepared as in Example 11 was tested on four herbicides, 2,4-D, (R,S)-dichlorprop, (R,S)-haloxyfop and (R)-haloxyfop (all at a final concentration of 0.5 mM) using 3 µl (42 µg) of *E. coli* extract per assay with a 15 min assay period. **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. **Table 33** shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

Table 33. Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

				A510	
STRUCTURE	Reg ID	Compound	Enantiomer	AAD1	AAD2
	18706	quizalofop	R	0.27	0.01
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15
	11044492	fenoxaprop	R	0.14	0
	43865	haloxyfop-acetate	--	0	0.22

[00442] The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. *See* **Figure 19.** The K_m values for 2,4-D were 97 and 423 μM for AAD-1 (v1) and AAD-2 (v1) respectively and the apparent V_{max} values were 0.11 and 0.86 A_{510} units, respectively (**Table 34**). Because equivalent amounts of enzyme were used in the assays (as determined by SDS-PAGE analysis), it can be concluded that the k_{cat} of AAD-2 (v1) for 2,4-D is almost 8-fold higher than AAD-1 (v1) and the k_{cat}/K_m is 2-

fold higher. Thus AAD-2 (v1) is significantly more efficient at cleaving 2,4-D *in vitro* than AAD-1 (v1). This is in surprising contrast to the *in planta* findings reported below, where plants expressing AAD-1 (v1) are significantly better in conferring 2,4-D resistance relative to AAD-2 (v1).

Table 34. Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ±SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes: Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

[00443] 17.4 – Evaluation of transformed *Arabidopsis*.

[00444] Freshly harvested T_1 seed transformed with a native [*AAD-1 (v2)*], plant optimized [*AAD-1 (v3)*], or native *AAD-2 (v1)* gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00445] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet and then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00446] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and

plants moved to the greenhouse (22±5° C, 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)*, *AAD-1 (v2)*,or *AAD-2 (v1)* to provide phenoxy auxin herbicide resistance.

[00447] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

[00448] Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0).

17.5 – Results of selection of transformed plants.

The first *Arabidopsis* transformations were conducted using *AAD-1 (v3)*. T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (*PAT* gene), equating to a transformation/ selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see **Table 15** in Example 6.5 above). A small lot of native *AAD-1 (v2)* transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency). Surprisingly, *Arabidopsis* transformations using the native *AAD-2(v1)* gene provided a very low transformation frequency when selected for glufosinate tolerance (*PAT* selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered, equating to a transformation/selection frequency of 0.018% (*see* **Table 15**). Transformation frequency for native *AAD-2 (v1)* was only 6% of that of native *AAD-1 (v2)*. The native *AAD2 (v1)* gene was subsequently synthetically optimized, cloned, and transformed as pDAB3705, into Arabidopsis using methods previously described (see Example 5). The plant optimized *AAD2 (v2)* (SEQ ID NO:29, which encodes SEQ ID NO:30) yielded a normal T_1 *Arabidopsis* selection frequency using Liberty herbicide of approximately 0.11% (*see* **Table 15**).

[00449] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** (in

Example 6.5 above) compares the response of *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* and *AAD-1(v2)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* or *AAD-2 (v2)* versus the wildtype or *PAT/Cry1F* transformed controls.

[00450] Surprisingly, *AAD-2 (v1)* transformants were far less resistant to 2,4-D than either *AAD-1 (v2)* or *AAD-1 (v3)* genes (**Table 16**) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) *AAD-1 (v2)*-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average = 34%), and >73% (11 of 15) *AAD-1 (v3)* T_1 plants were uninjured (population injury average = 14%). *See* **Figure 20**. Tolerance improved slightly for plant-optimized *AAD2 (v2)* versus the native gene; however, comparison of both *AAD-1* and *-2* plant optimized genes indicates a significant advantage for *AAD-1 (v3) in planta* (see **Table 16**).

[00451] These results are unexpected given that *in vitro* comparison of native AAD-1 (v2) and AAD-2 (v1) indicated 2,4-D was better degraded by AAD-2 (v1). AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. *See* **Figure 21**. No large difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes. These data corroborate earlier findings that make the functional expression of AAD-1 (v3) *in planta* for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected .

Example 18 – Preplant Burndown Applications

[00452] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

[00453] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (*see* federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With *AAD-1 (v3)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will

be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed.

Example 19 – In-Crop Use of Phenoxy Auxins Herbicides in *AAD-1 (v3)* Only Transformed Soybeans, Cotton, and Other Dicot Crops

[00454] *AAD-1 (v3)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D, dichlorprop, MCPA, *et al.*) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot crops which are naturally tolerant to these herbicides. A combination of quizalofop (20 – 100 g ae/ha) plus 2,4-D (420 – 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)*-transformed dicot crop (*i.e.*, soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (*see* weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00455] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if

broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

[00456] Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of *AAD-1 (v3)*-transformed dicot crop volunteers.

[00457] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - In-Crop Use of Phenoxy Auxins and AOPP Herbicides in *AAD-1 (v3)* Only Transformed Corn, Rice, and Other Monocot Species

[00458] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-1 (v3)* would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. *AAD-1 (v3)*-transformed monocot crops would, therefore,

enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 – 1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, *et al.* could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of *AAD-1 (v3)*-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20 – 100 g ae/ha) plus 2,4-D (420 – 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)* transformed monocot crop (*e.g.*, corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00459] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-

crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

[00460] Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the *AAD-1 (v3)* gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-1 (v3)* Stacked With Glyphosate Tolerance Trait in Any Crop

[00461] The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-1 (v3)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with*AAD-1 (v3)*, one can selectively

apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a GT trait are stacked in any monocot or dicot crop species:

a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (*e.g.*, *Commelina* spp), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-1 (v3)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

[00462] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or

independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 22 - *AAD-1 (v3)* Stacked with Glufosinate Tolerance Trait in Any Crop

[00463] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

[00464] By stacking *AAD-1 (v3)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and

broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant grass weed species (e.g., *Echinochloa spp* or *Sorghum spp*) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00465] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 23 - *AAD-1 (v3)* Stacked with *AHAS* Trait in Any Crop

[00466] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone

herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking *AAD-1 (v3)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

   ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea spp.* can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

   iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00467] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by *AAD-1 (v3)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 24 – *AAD-1 (v3)* in Rice

[00468] 24.1 – Media description.

[00469] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

[00470] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB

medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3–4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (1/2MSH50).

[00471] 24.2 – Tissue culture development.

[00472] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then

moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week prior to storage at 4° C.

[00473] 24.3 – Microprojectile bombardment.

[00474] All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB3403, 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[00475] 24.4 – Tolerance testing.

[00476] Rice plantlets at the 3-5 leaf stage were sprayed with a 0.3% (v/v) solution of DuPont™ Assure® II containing 1% (v/v) Agridex crop oil concentrate using a DeVilbiss bulb sprayer (model 15-RD glass atomizer). This concentration corresponds to approximately 140 g ae/ha. Each plant was spayed in a fume hood at a distance of 8-12 inches with 6 full squirts of the sprayer directed so that the entire plant was covered with an equal portion of herbicide. Each squirt delivered approximately 100 µl solution to the plantlet. Once sprayed, plantlets were allowed to dry for one hour before being moved out of the fume hood. Rating for sensitivity or resistance was done at 10-14 days after treatment (DAT) and is shown in **Table 35** below.

Table 35.

Sample Name	**140 g ae/ha quizalofop**
Control	Dead
63-1A	No injury
63-1F	No injury
63-4B	No injury
63-4D	No injury
63-6C	Dead

[00477] 24.5 – Tissue harvesting, DNA isolation and quantification.

[00478] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

[00479] 24.6 – Southern blot analysis.

[00480] Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 ug of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 x SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80°C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward - ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse - GGGCAGGCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute and 45 seconds followed by 72°C for 10 minutes.

[00481] The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60°C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it

RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65°C in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in **Table 36.**

Table 36. Southern Results.

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

[00483] Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

[00484] 24.7 – Western data

[00485] Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in **Table 37.**

Table 37.

Lane	Sample Name	TSP (µg/mL)	ELISA [AAD1] (ng/mL)	ELISA Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	-
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	-
7	AAD1 Standard (0.5 µg/mL)				+++
8	AAD1 Standard (5.0 µg/mL)				+++++

Example 25 – Turf Grass Transformation Procedures

[00486] Genetic transformation, with *AAD-1 (v3)* substituted for the "bar" gene as described below, of creeping bentgrass mediated by Agrobacterium tumefaciens could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. *See* "Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation" (Luo *et. al.*, 2004).

[00487] Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T_1 generation. All independent transformation events carried one to three copies of the transgene, and a majority (60–65%) contained only a single copy of the foreign gene with no apparent rearrangements.

[00488] 25.1 – Seed preparation for embryogenic callus induction.

[00489] Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v) Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-*o*-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120°C for 20 min).

[00490] 25.2 – Embryogenic callus induction.

[00491] The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

[00492] 25.3 – *Agrobacterium* infection and co-cultivation.

[00493] One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 µM acetosyringone. A 10-ul aliquot of Agrobacterium (LBA4404) suspension (OD=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25°C.

[00494] 25.4 – Resting stage and *Agrobacterium* control.

[00495] For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

[00496] 25.5 – Selection and potential transgenic colony identification.

[00497] Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

[00498] 25.6 – Regeneration of transgenic plants.

[00499] For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2–3 weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

[00500] 25.7 – Root induction and transfer to greenhouse.

[00501] Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3–5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

[00502] 25.8 – Vernalization and out-crossing of transgenic plants.

[00503] Transgenic plants were maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25°C under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3-4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25°C, and T1 plants were grown in the greenhouse for further analysis.

[00504] 25.9 – Other target grasses.

[00505] Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutate*), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

Example 26 – *AAD-1 (v3)* in Canola

[00506] 26.1 – Canola transformation.

[00507] The *AAD-1 (v3)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation. The construct contained *AAD-1 (v3)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

[00508] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00509] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00510] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the

segments were placed K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00511] Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of *AAD-1 (v3)*,whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the AAD-1 protein as determined by ELISA (section on protein analysis).

[00512] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 3 weeks.

[00513] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00514] The transformed T0 plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the *AAD-1 (v3)* gene.

[00515] 26.2 – "Molecular analysis": canola materials and methods

[00516] 26.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green

(Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/ul.

[00517] 26.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-1 (v3)* were (Forward – ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse - CGGGCAGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-1 (v3)* events tested positive. Three negative control samples tested negative.

[00518] 26.3 – ELISA.

[00519] Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in **Table 38.**

Table 38. Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (µg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	-
Control 2	61	2549.60	35.00	13.73	-
Control 3	59	2374.41	22.79	9.60	-

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Zhao, Z.Y., T. Cai, L. Tagliani, M. Miller, N. Wang, H. Pang, M. Rudert, S. Schroeder, D. Hondred, J. Seltzer, and D. Pierce. 2000. *Agrobacterium*-mediated sorghum transformation, Plant Mol. Biol. 44:789-798.

Zipper, C., M. Bunk, A. Zehnder, H. Kohler. 1998. Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(RS)-2-(2,4-dichlorophenoxy)propanoic acid] by Sphingobium herbicidovorans MH. J. Bact. 13:3368-3374

Claims

Claim 1.

A transgenic plant cell comprising a polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO:9 or a variant of SEQ ID NO:9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO:9.

Claim 2.

The cell of claim 1 wherein said plant cell is selected from the group consisting of dicotyledonous cells and monocotyledonous cells.

Claim 3.

The cell of claim 2 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

Claim 4.

The cell of claim 2 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

Claim 5.

A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.

Claim 6.

The plant of claim 5 wherein said herbicide is a phenoxy auxinic herbicide.

Claim 7.

The plant of claim 5 wherein said herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

Claim 8.

The plant of claim 5 wherein said herbicide is an aryloxyphenoxypropionate.

Claim 9.

The plant of claim 5 wherein said herbicide is selected from the group consisting of fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

Claim 10.

The plant of claim 5 wherein expression of said polynucleotide renders said plant resistant to both a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

Claim 11.

The plant of claim 5 wherein said plant further comprises a second herbicide resistance gene.

Claim 12.

The plant of claim 11 wherein said second herbicide resistance gene renders said plant resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), and inhibitors of protoporphyrinogen oxidase (PPO).

Claim 13.

A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 5, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

Claim 14.

The method of claim 13 wherein said herbicide is an R-enantiomer of a chiral phenoxy auxin.

Claim 15.

The method of claim 14 wherein said herbicide is selected from the group consisting of R-dichlorprop and R-mecoprop.

Claim 16.

The method of claim 13 wherein said herbicide is an achiral phenoxy auxin.

Claim 17.

The method of claim 16 wherein said herbicide is selected from the group consisting of 2,4-D and MCPA.

Claim 18.

The method of claim 13 wherein said first herbicide is an aryloxyphenoxypropionate and said plant is a monocot.

Claim 19.

The method of claim 18 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, sorghum, and pasture grasses.

Claim 20.

The method of claim 13 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

Claim 21.

The method of claim 20 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

Claim 22.

The method of claim 13 wherein said method comprises applying a second herbicide.

Claim 23.

The method of claim 22 wherein said first herbicide and said second herbicide are applied sequentially.

Claim 24.

The method of claim 22 wherein said first herbicide and said second herbicide are applied concurrently.

Claim 25.

The method of claim 22 wherein said first herbicide is a phenoxy auxin and second herbicide is an aryloxyphenoxypropionate.

Claim 26.

The method of claim 13 wherein said plant is resistant to glyphosate.

Claim 27.

The method of claim 14 wherein said R-enantiomer is a component of a racemic mixture.

Claim 28.

The method of claim 22 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

Claim 29.

The method of claim 28 wherein said second gene is selected from the group consisting of a modified AHAS (acetohydroxyacid synthase), SurA, SurB, Csr1, Csr1-1, Csr1-2, a modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT, PAT (phosphinothricin-N-acetyltransferase), bar, and a dicamba-degrading enzyme.

Claim 30.

The method of claim 28 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase inhibitors, protoporphyrinogen oxidase inhibitors, and hydroxyphenyl-pyruvate-dioxygenase inhibitors.

Claim 31.

The method of claim 30 wherein said second herbicide is an acetolactate synthase inhibitor selected from the group consisting of imidazolinones, sulfonyl ureas, and triazolopyrimidine herbicides.

Claim 32.

The method of claim 31 wherein said second herbicide is an imidazolinone selected from the group consisting of imazamox, imazethapyr, imazaquin, and imazapic.

Claim 33.

The method of claim 28 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

Claim 34.

The method of claim 33 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

Claim 35.

The method of claim 28 wherein said first herbicide is an aryloxyphenoxypropionate and said second herbicide is glyphosate.

Claim 36.

The method of claim 35 wherein said first herbicide is selected from the group consisting of quizalofop, haloxyfop, and cyhalofop.

Claim 37.

The method of claim 31 wherein said second herbicide is a sulfonyl urea selected from the group consisting of amidosulfuron, bensulfuron, chlorimuron, chlorsulfuron, cinosulfuron, flupyrsulfuron, foramsulfuron, halosulfuron, nicosulfuron, primisulfuron, prosulfuron, rimsulfuron, sulfometuron, sulfosulfuron, thifensulfuron, triasulfuron, trifloxysulfuron, and triflusulfuron.

Claim 38.

The method of claim 28 wherein said method further comprises applying a third herbicide.

Claim 39.

The method of claim 38 wherein said herbicides are 2,4-D; quizalofop; and glufosinate.

Claim 40.

A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said first herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate.

Claim 41.

The method of claim 40 wherein said first herbicide is an acid, an inorganic salt, an organic salt, an ester, an R-enantiospecific isomer, or a component of a racemic mixture.

Claim 42.

The method of claim 40 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

Claim 43.

The method of claim 42 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate

Claim 44.

A polynucleotide optimized for expression in a plant wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

Claim 45.

The polynucleotide of claim 44 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

Claim 46.

The polynucleotide of claim 44 wherein said polynucleotide comprises SEQ ID NO:5.

Claim 47.

An isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell.

Claim 48.

The polynucleotide of claim 47 wherein said promoter is a plant promoter.

Claim 49.

The polynucleotide of claim 47 wherein said promoter is a cassava vein mosaic virus promoter.

Claim 50.

A method of selecting for a transformed plant cell wherein said method comprises subject a plurality of plant cells to transformation with a polynucleotide of claim 44 or 47 and growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxyalkanoate.

Claim 51.

The method of claim 50 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

Claim 52.

A polynucleotide of claim 47 or 50 wherein said polynucleotide is used as a selectable marker in the method of claim 50.

Claim 53.

A method of detecting whether a plant comprises a polynucleotide of claim 47 or 50 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

Claim 54.

The method of claim 53 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

Claim 55.

The method of 53 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

Claim 56.

The method of 54 wherein said method comprises using an antibody to detect for the presence of said protein.

Claim 57.

A seed comprising a plant cell of claim 1.

Claim 58.

A plant grown from the seed of claim 57.

Claim 59.

A part, progeny, or asexual propagate of the plant of claim 5.

Claim 60.

The method of claim 13 wherein said method is used to treat or prevent herbicide-resistant weeds.

Claim 61.

The plant of claim 5 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

Claim 62.

The plant of claim 5 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

Claim 63.

A method of conferring 2,4-D herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a nucleic-acid-encoding enzyme having R-specific aryloxyalkanoate dioxygenase activity relative to a 2,4-D herbicide.

Claim 64.

A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 47, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

Claim 65.

The method of claim 64 wherein said herbicide is a phenoxy auxin.

Claim 66.

The method of claim 64 wherein said herbicide is an aryloxy phenoxypropionate.

Claim 67.

The method of claim 64 wherein said plants are dicots.

Claim 68.

The method of claim 64 wherein said plants are monocots.

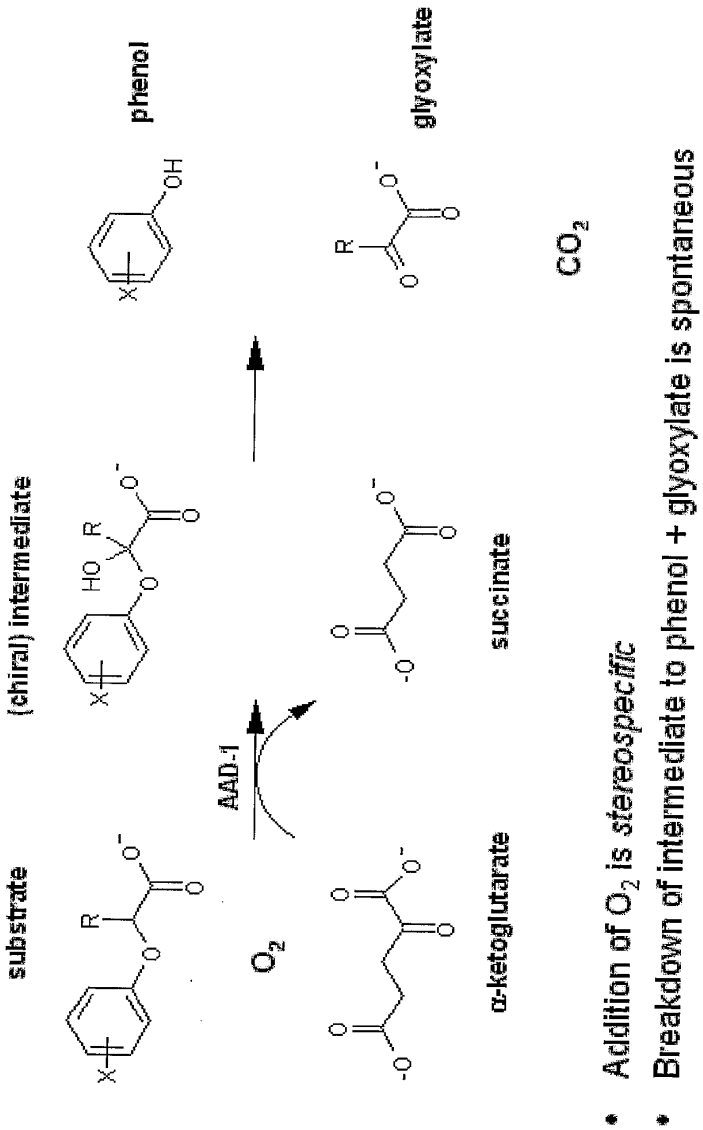

substrate
(chiral) intermediate
phenol
glyoxylate
AAD-1
O_2
α-ketoglutarate
succinate
CO_2
• Addition of O_2 is *stereospecific*
• Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

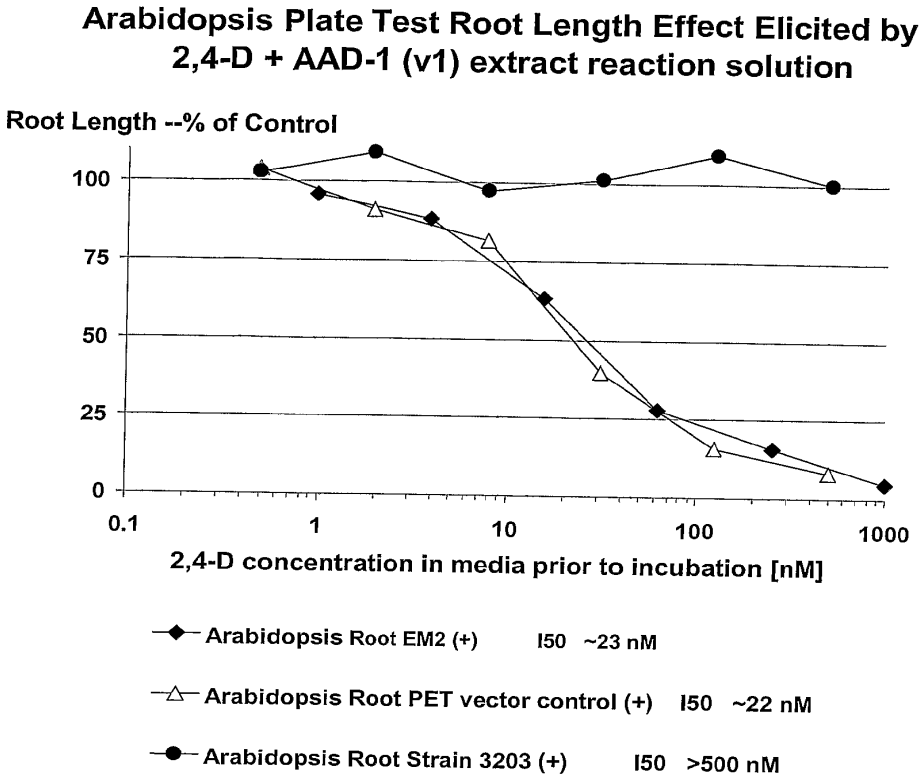

Arabidopsis Plate Test Root Length Effect Elicited by 2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

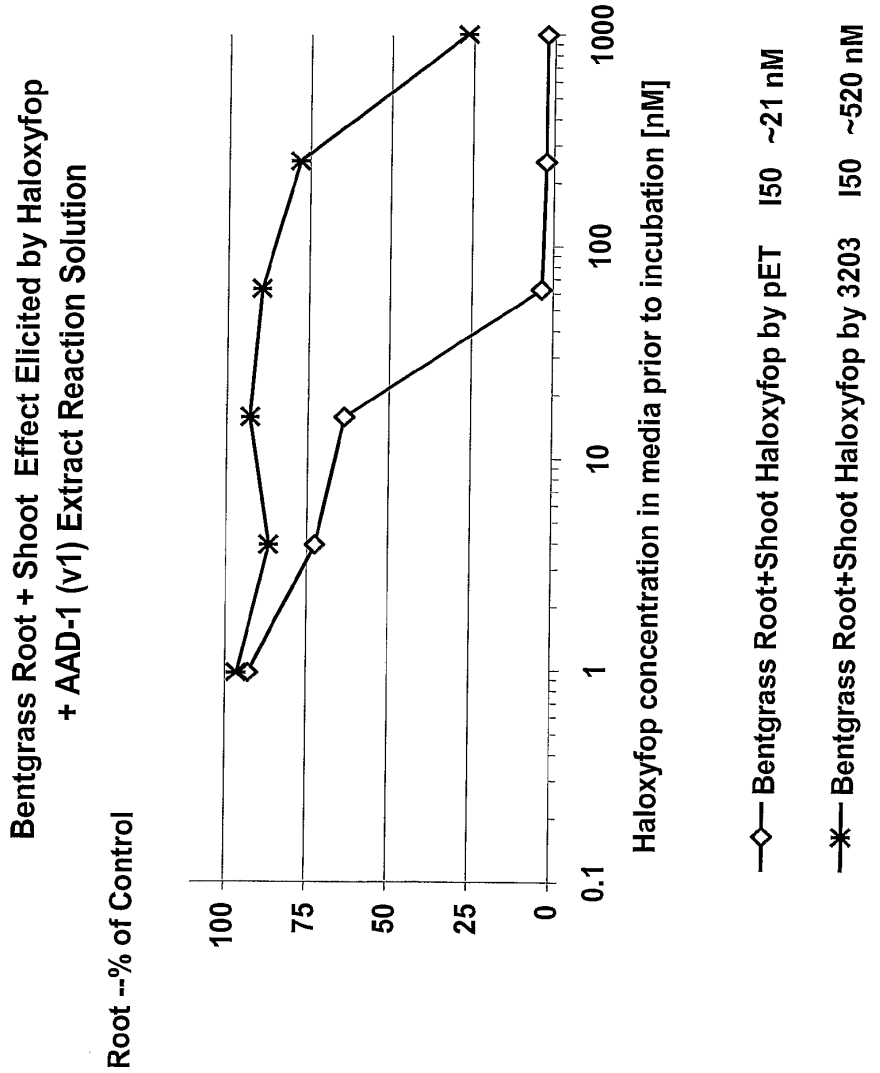

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

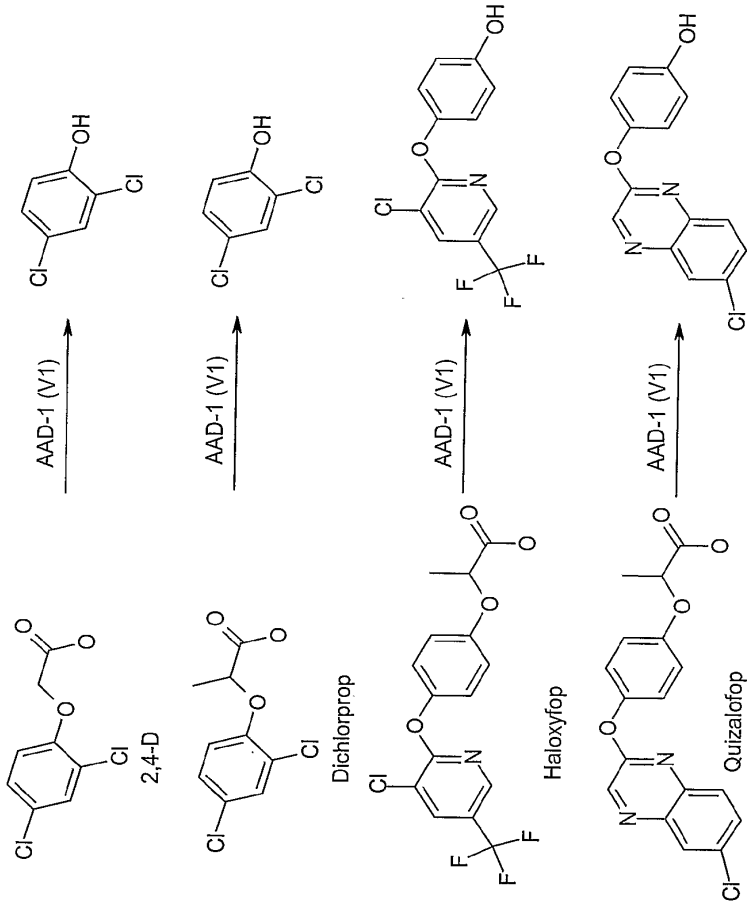

AAD-1 (V1)
2,4-D
AAD-1 (V1)
Dichlorprop
AAD-1 (V1)
Haloxyfop
AAD-1 (V1)
Quizalofop

Fig. 4

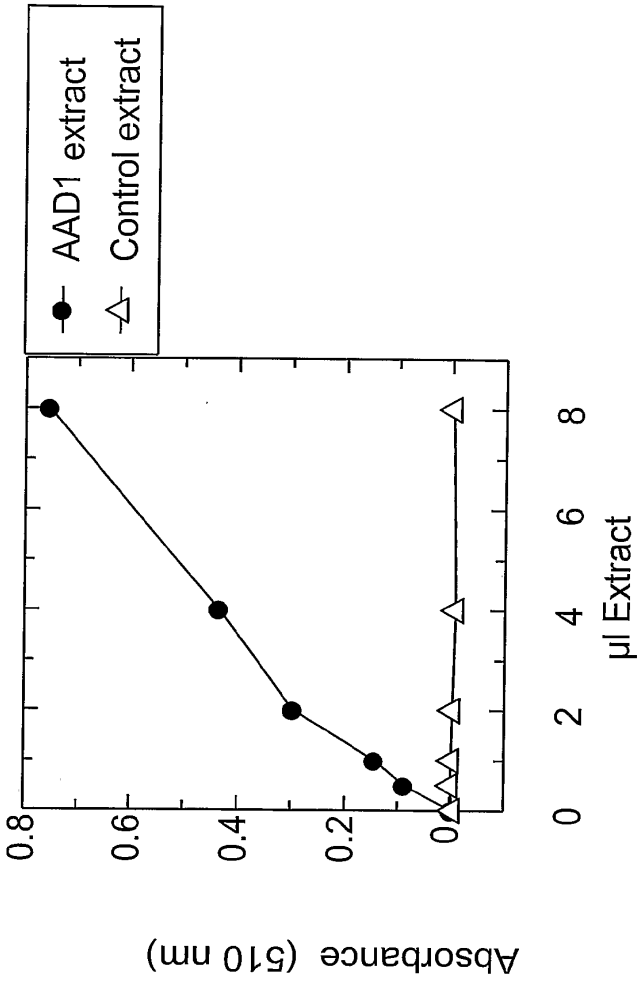

AAD1 extract
Control extract
Absorbance (510 nm)
0.8
0.6
0.4
0.2
0
0
2
4
6
8
µl Extract

Fig. 5

Fig. 6A

Fig. 6B

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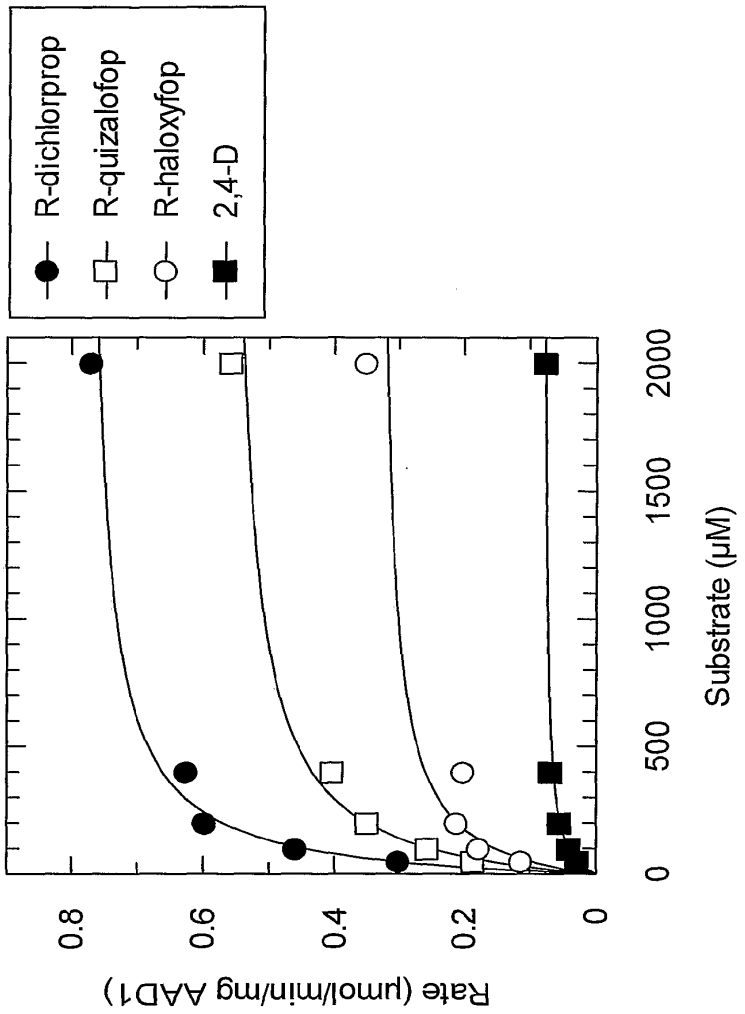

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

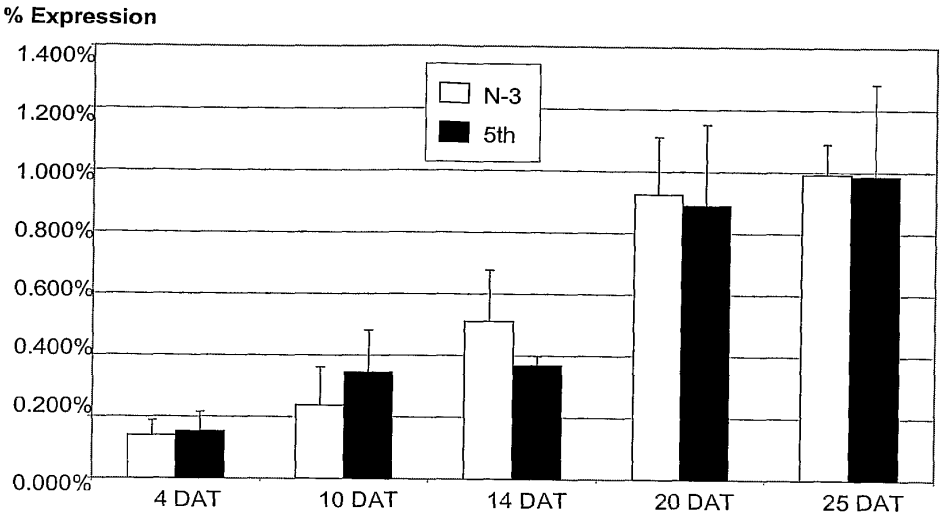

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

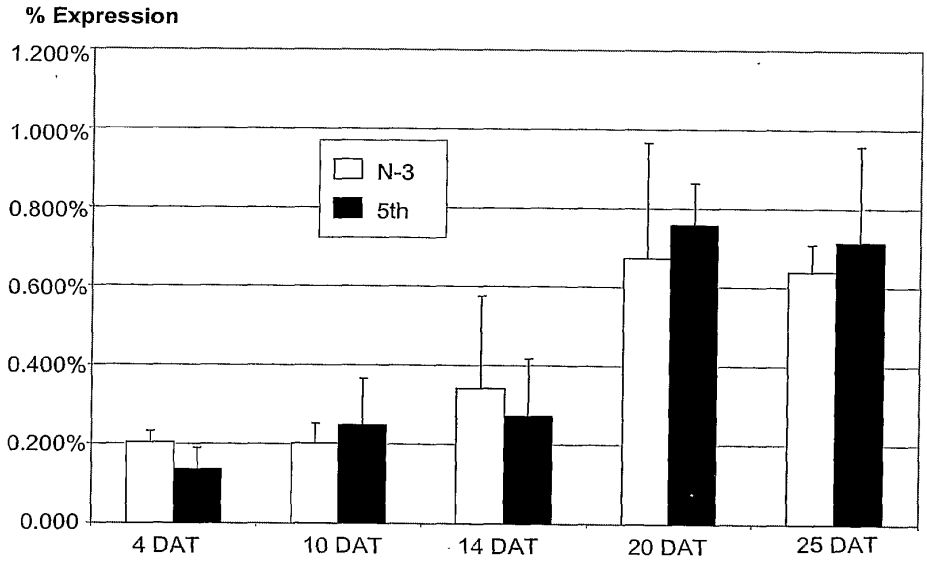

% Expression
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

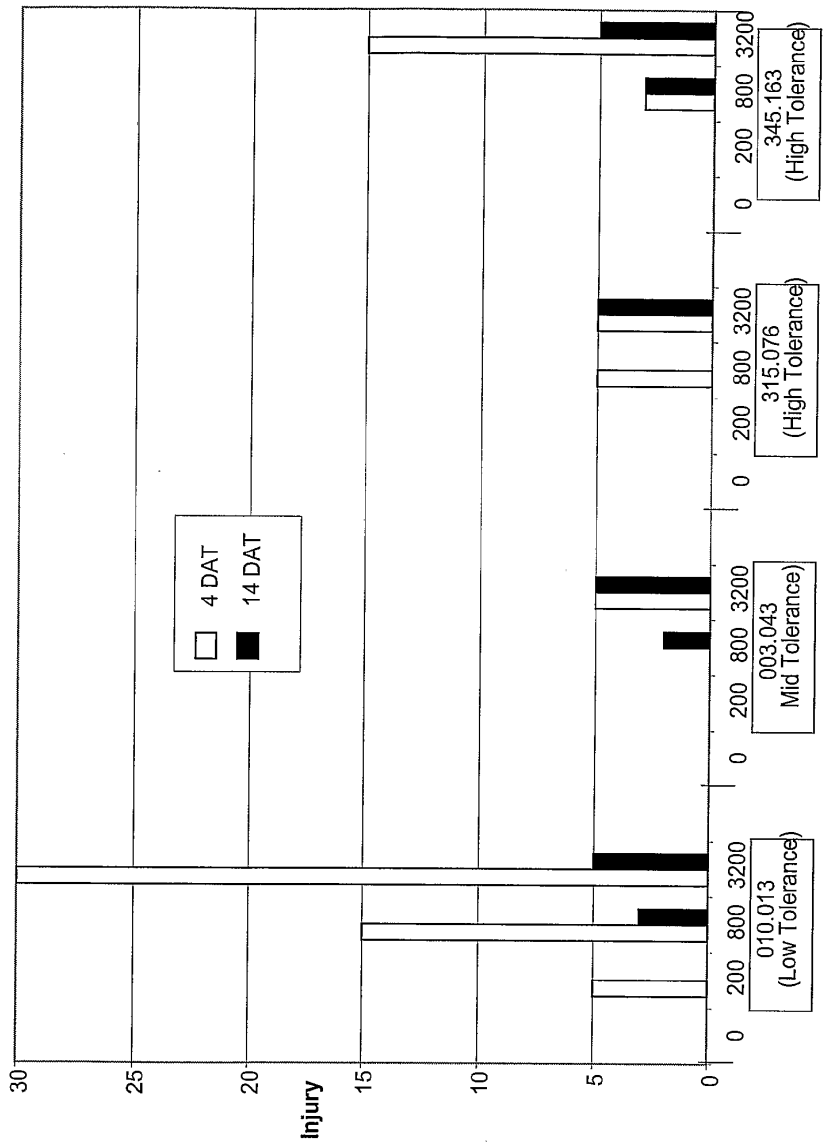

4 DAT
14 DAT
Injury
0
5
10
15
20
25
30
0 200 800 3200
010.013
(Low Tolerance)
0 200 800 3200
003.043
Mid Tolerance)
0 200 800 3200
315.076
(High Tolerance)
0 200 800 3200
345.163
(High Tolerance)

Fig. 9A

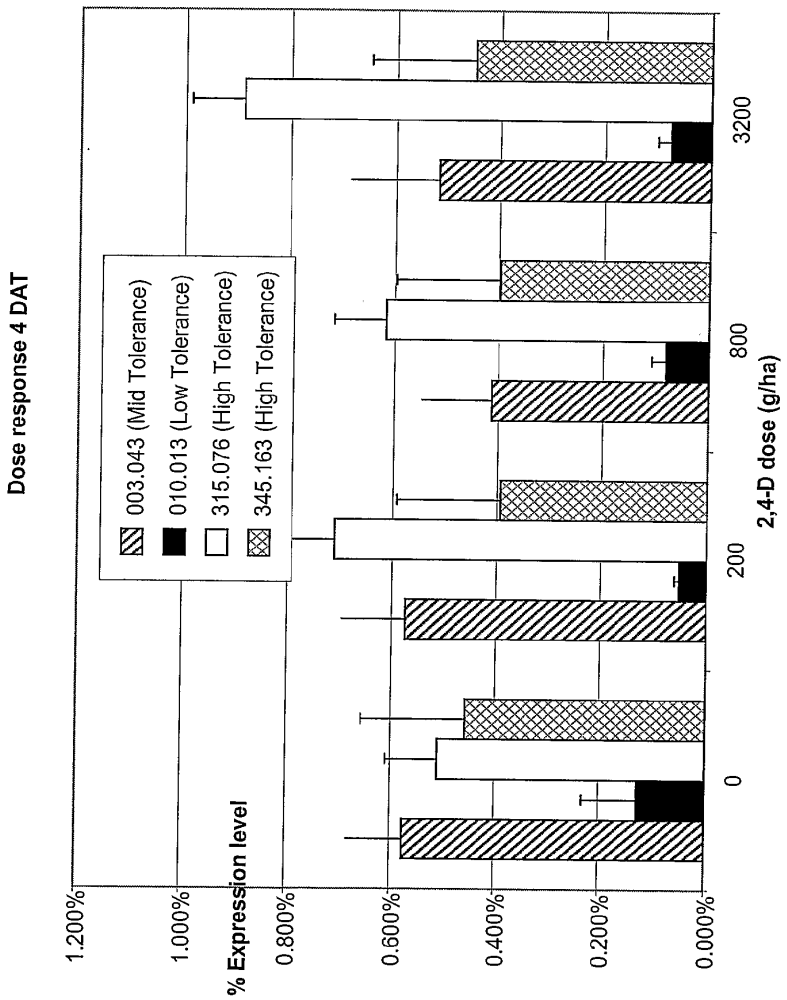

Dose response 4 DAT
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

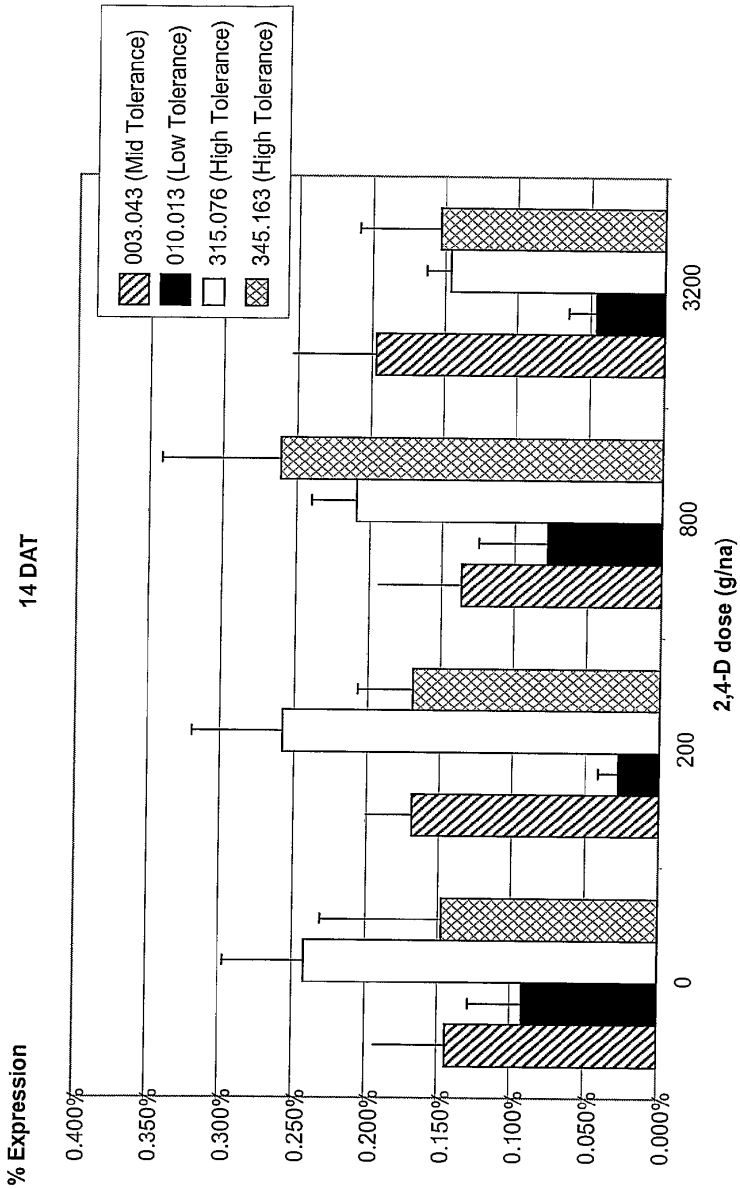

003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
14 DAT
% Expression
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

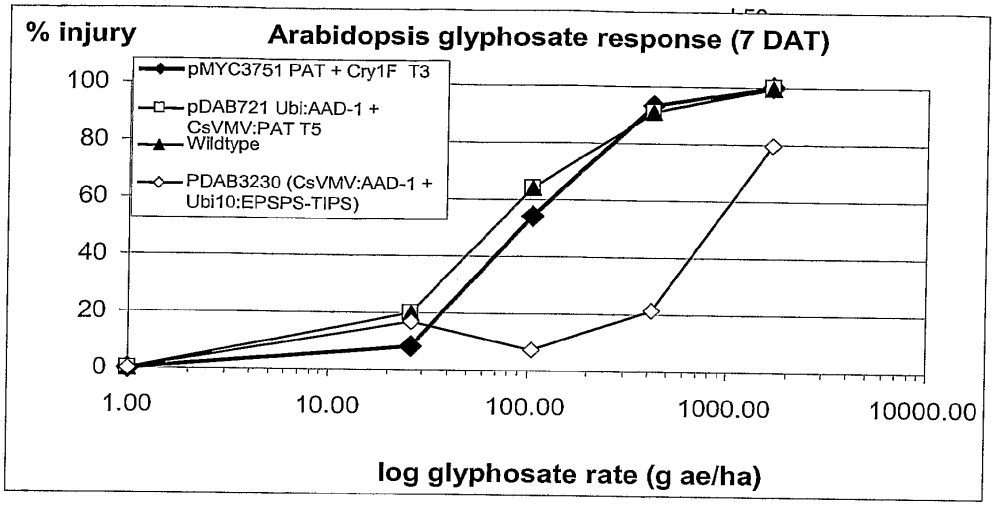

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5 Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

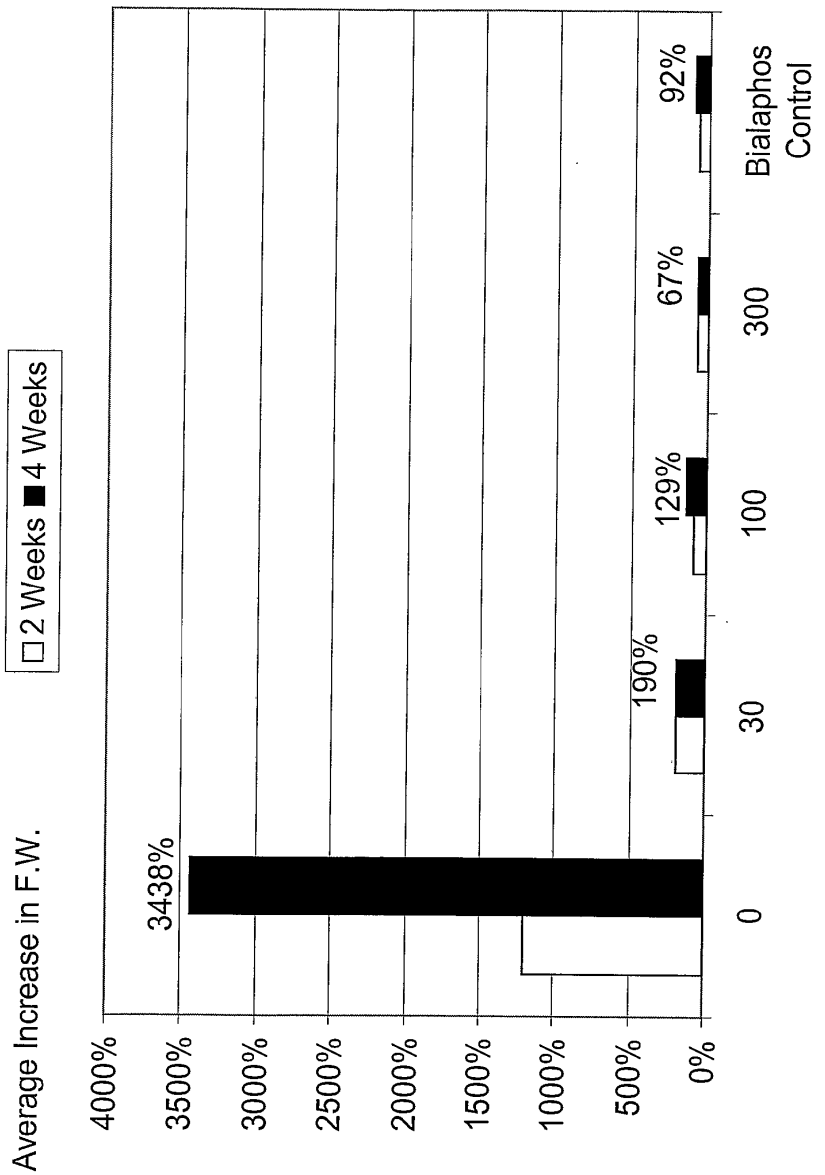

Average Increase in F.W.
□2 Weeks ■4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

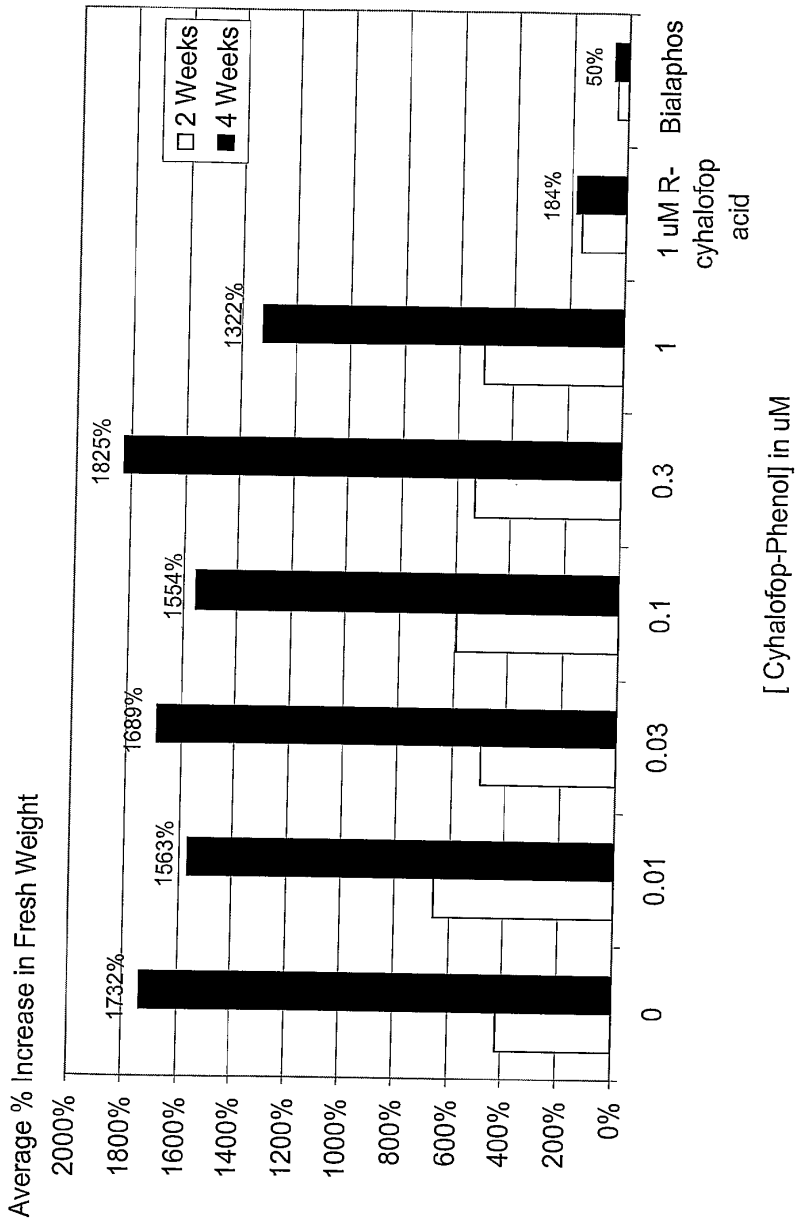

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
2 Weeks
4 Weeks
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

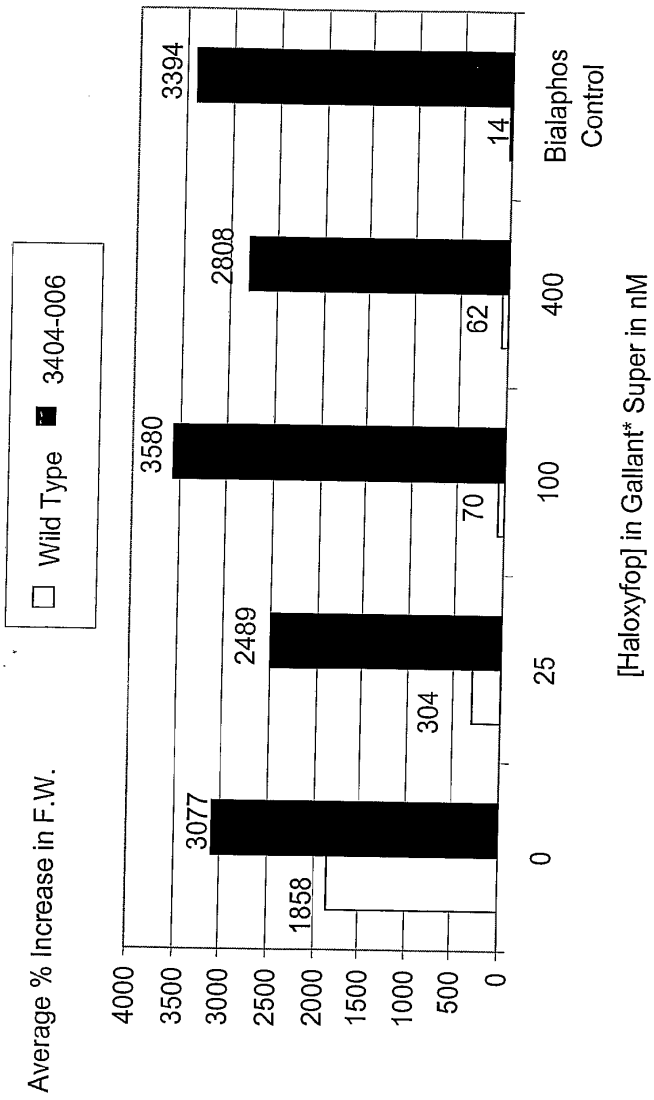

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
3077
1858
2489
304
3580
70
2808
62
3394
14
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant* Super in nM

Fig. 13

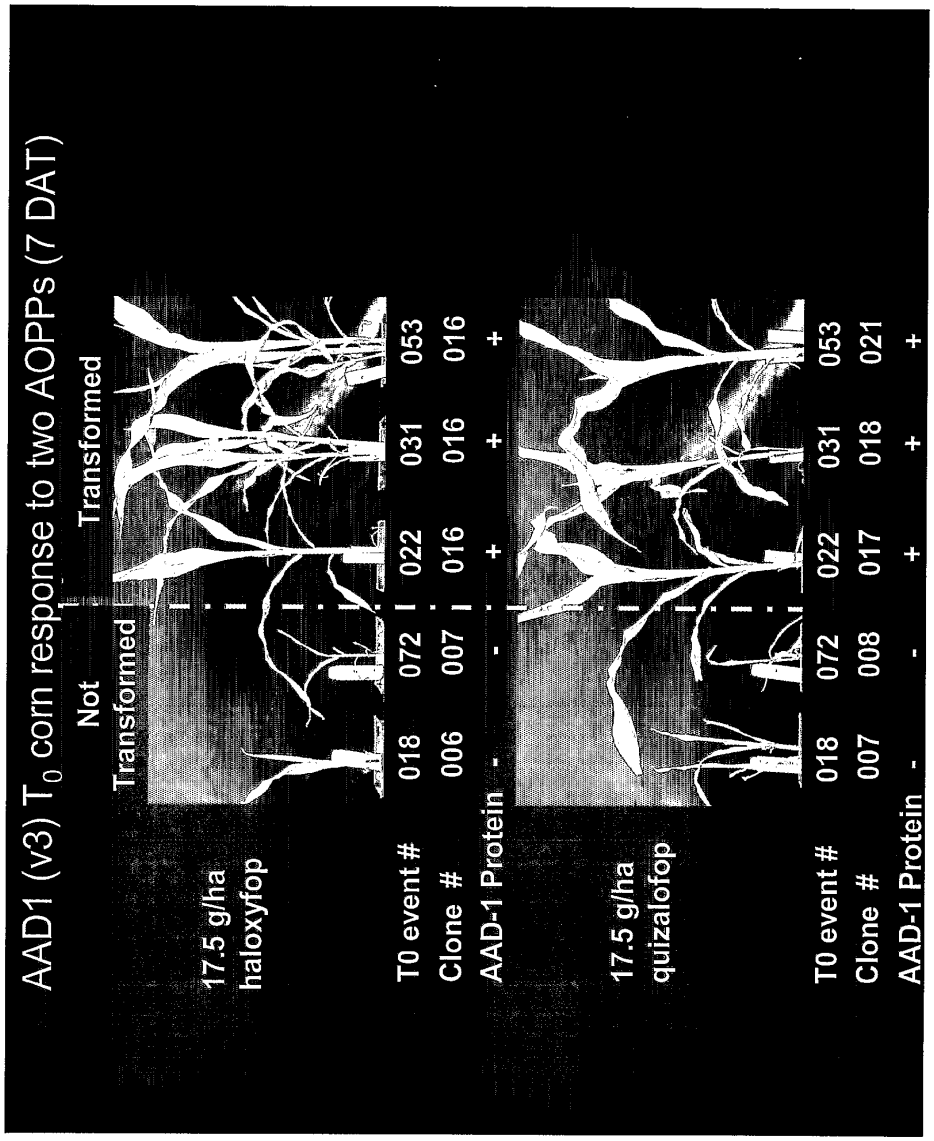

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

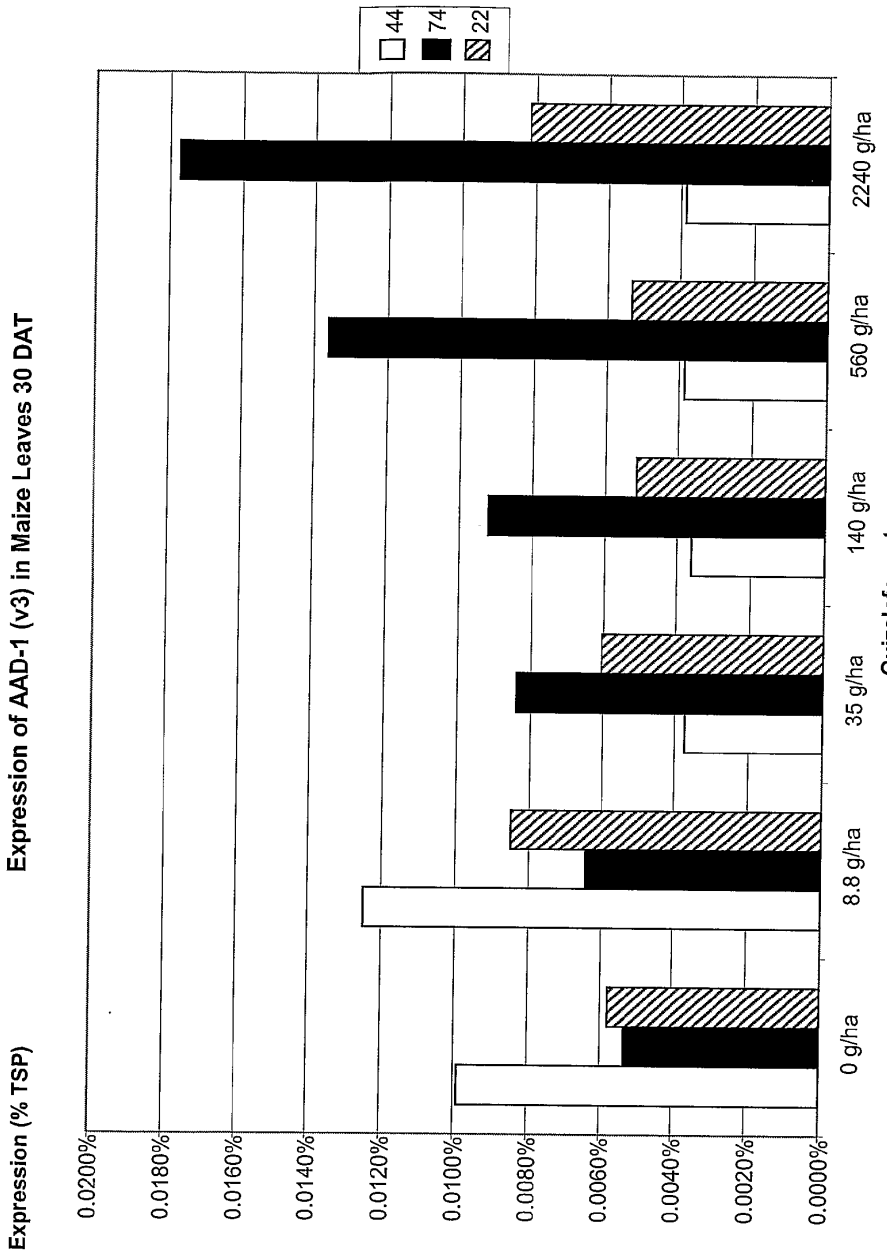

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
44
74
22
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate

Fig. 15

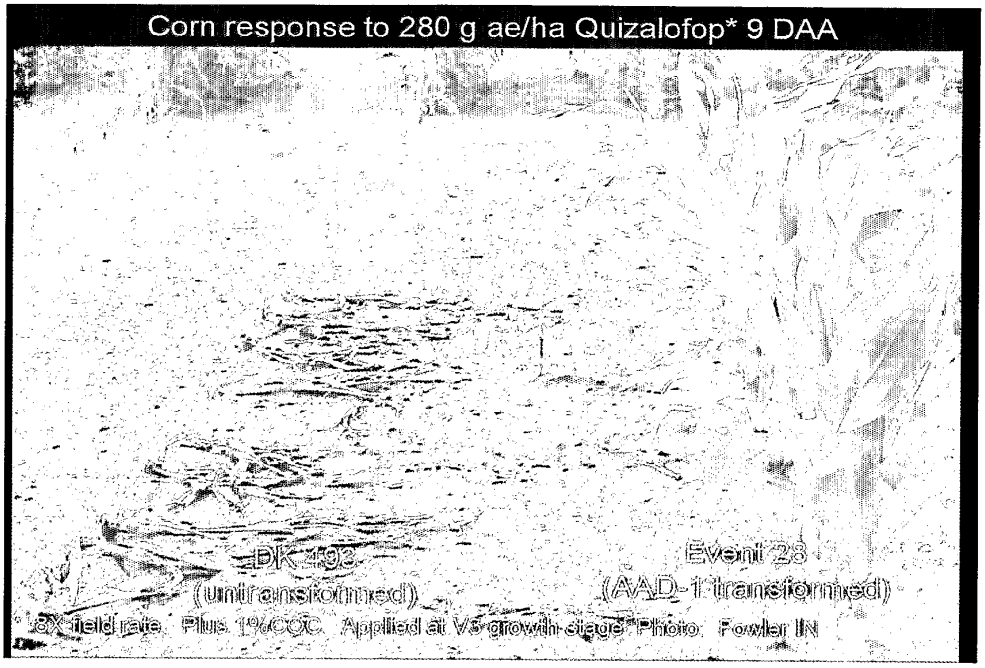

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 493
(untransformed)
Event 28
(AAD-1 transformed)
8X field rate Plus 1%COC Applied at V5 growth stage Photo Fowler IN

Fig. 16

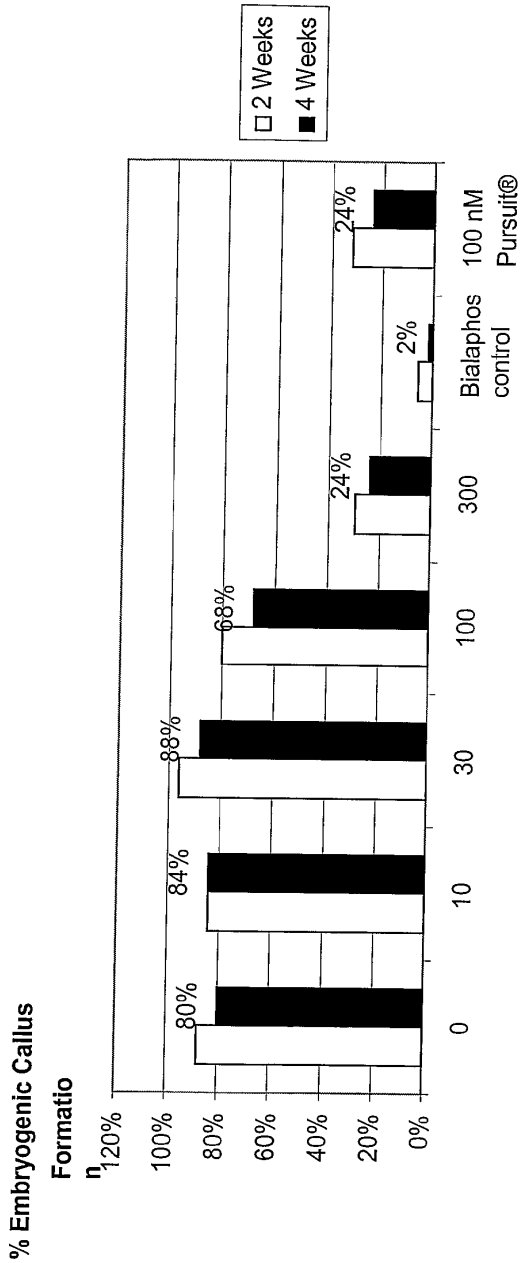
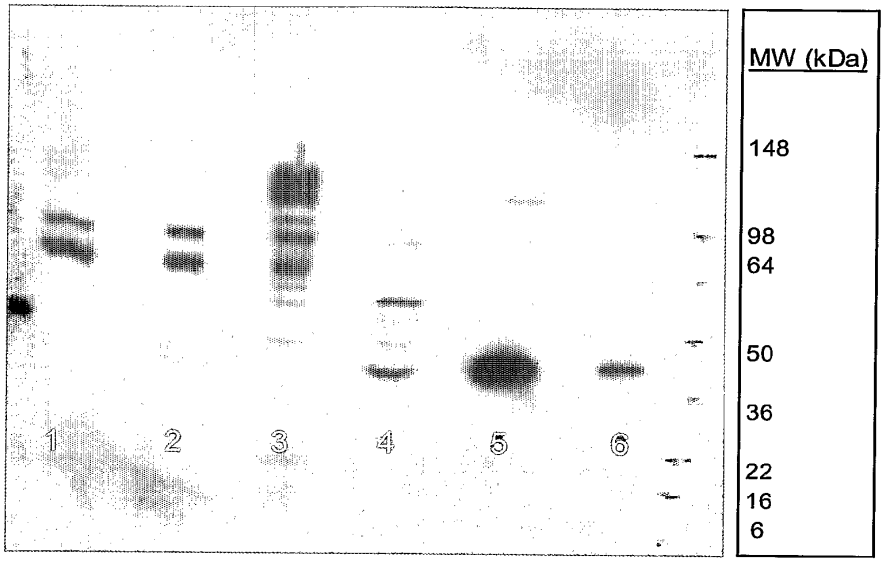

% Embryogenic Callus Formation
120%
100%
80%
60%
40%
20%
0%
2 Weeks
4 Weeks
80%
84%
88%
68%
24%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
[R-cyhalofop ACID] in nM

Fig. 17



Western

Lane	
1	Calli #1
2	Calli #2
3	Negative
4	AAD1 Corn
5	Standard 5 ug/mL
6	Standard 0.5 ug/mL

Fig. 18

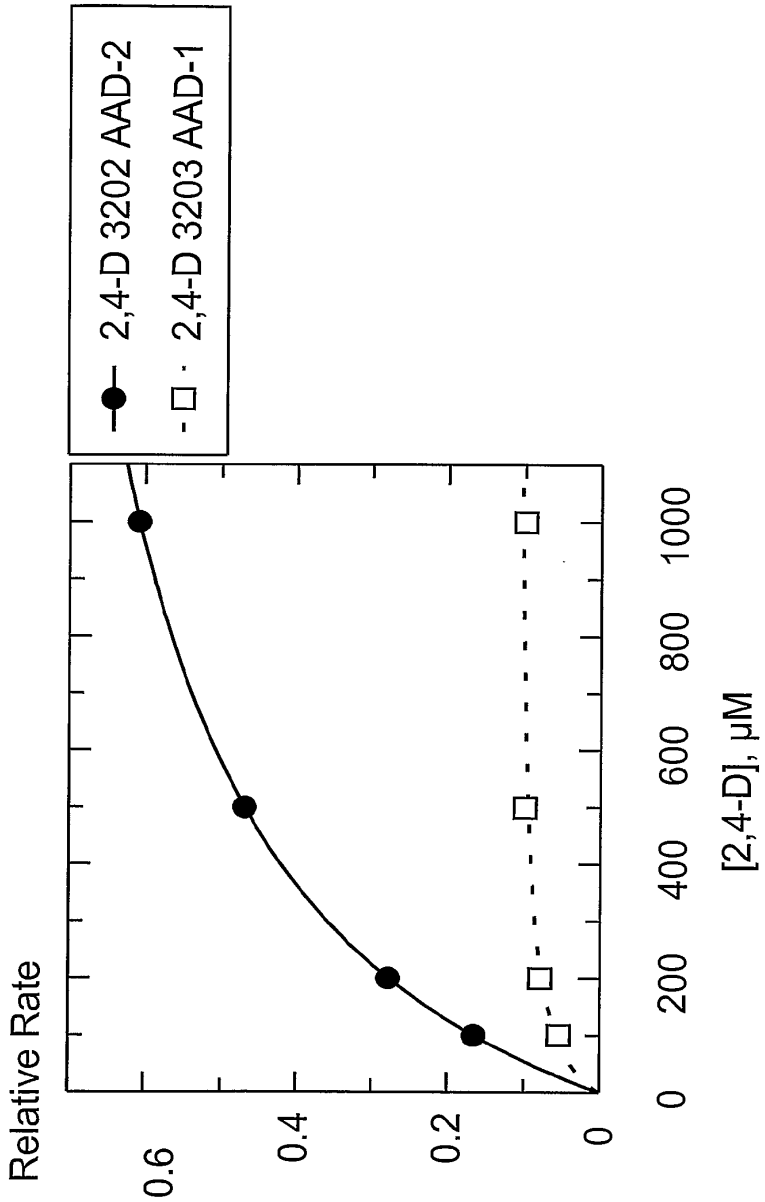

2,4-D 3202 AAD-2
2,4-D 3203 AAD-1
Relative Rate
0
0.2
0.4
0.6
0
200
400
600
800
1000
[2,4-D], µM

Fig. 19

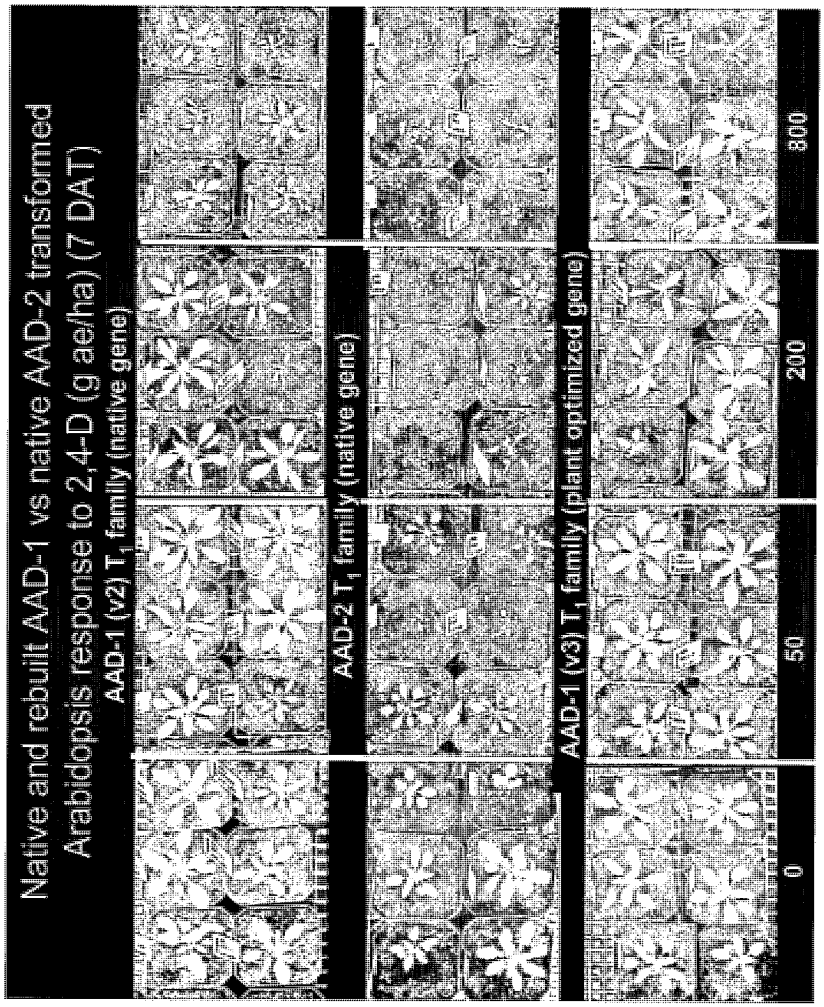

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

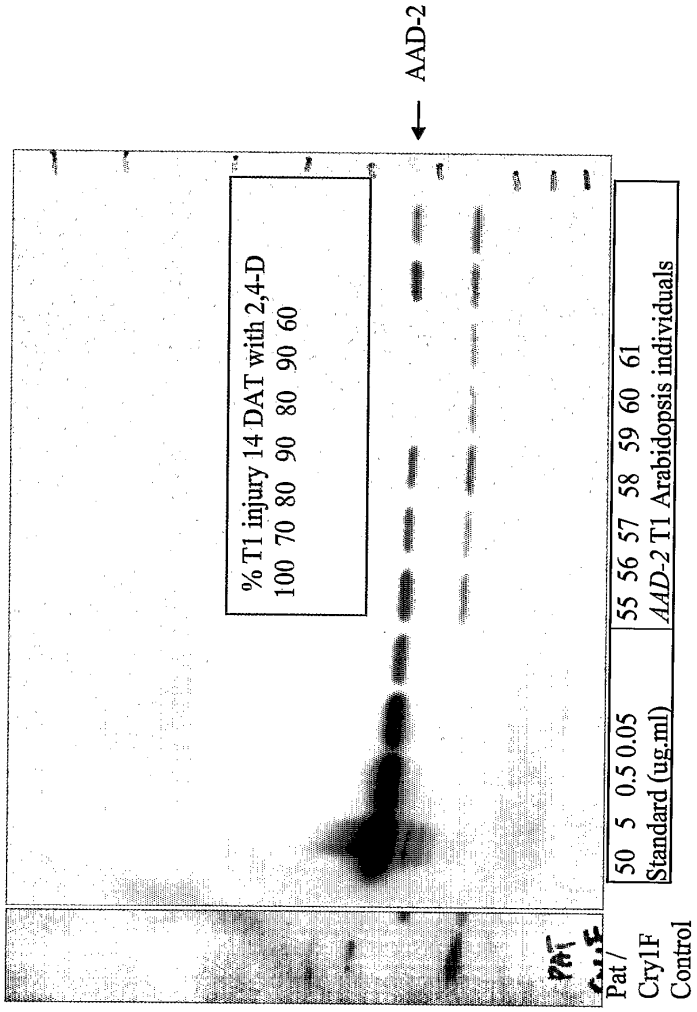

AAD-2
% T1 injury 14 DAT with 2,4-D
100 70 80 90 80 90 60
50 5 0.5 0.05
Standard (ug.ml)
55 56 57 58 59 60 61
AAD-2 T1 Arabidopsis individuals
Pat /
Cry1F
Control

Fig. 21

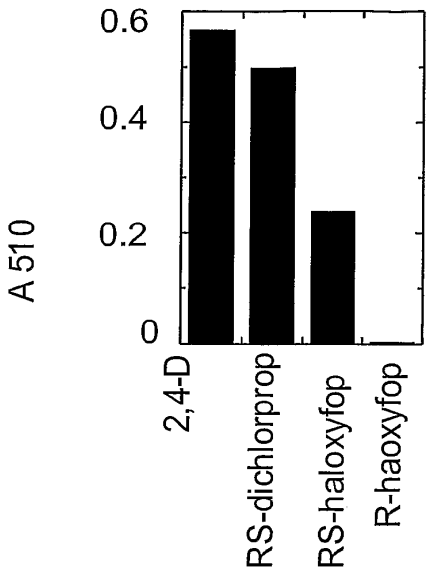


Fig. 22

SEQUENCE LISTING

<110> Dow AgroSciences LLC

<120> Novel Herbicide Resistance Genes

<130> DAS-115XC1

<150> US 60/567,052
<151> 2004-04-30

<160> 32

<170> PatentIn version 3.2

<210> 1
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer used to amplify the rdpA/AAD-1 (v1) gene

<400> 1
tctagaagga gatataccat gcatgctgca ctgtcccccc tctcccagcg 50

<210> 2
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer used to amplify the rdpA/AAD-1 (v1) gene

<400> 2
ctcgagttac tagcgcgccg ggcgcacgcc accgaccg 38

<210> 3
<211> 915
<212> DNA
<213> Sphingobium herbicidovorans

<220>
<221> misc_feature
<222> (1)..(18)
<223> Primer linker

<220>
<221> misc_feature
<222> (907)..(915)
<223> Primer linker

<400> 3
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atcgcggtcc agccgctgac cggcgtcctg ggcgccgaga tcaccggcgt cgacctgcgc 120

gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttccacac ttaccaggtc 180

atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc 240

ggccccgtcg atcccgtgcc cctgctcaag agcatcgaag ggtatccaga ggtgcagatg 300

atccgccgcg aagccaacga aagcgggcgt gtgatcggtg aygactggca caccgacagc 360

accttcctgg acgcaccgcc ggccgccgtg gtgatgcgcg cgatcgacgt gcccgagcat 420

ggcggcgaca ccggttttct gagcatgtac accgcgtggg agacgctgtc gcccaccatg 480

caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggctcgctc 540

taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg 600

ggcgaccgtg aaaccgtgca ccccctggtg gtgacccatc cgggcagcgg ccgcaagggc 660

ctgtacgtga accaggtcta ttgccagcgc atcgagggca tgaccgatgc cgaaagcaaa 720

ccgctgctgc agttcctgta cgagcatgcg acacggttcg atttcacctg ccgcgtgcgc 780

tggaagaagg accaggtcct ggtctgggac aacctgtgca cgatgcaccg ggccgtaccc 840

gactacgcgg gcaagttccg ctacctgacg cgcaccacgg tcggtggcgt gcgcccggcg 900

cgctagtaac tcgag 915

<210> 4
<211> 897
<212> DNA
<213> Artificial Sequence

<220>
<223> AAD-1 (v2) primary sequence

<220>
<221> misc_feature
<222> (1)..(2)
<223> primer linker

<220>
<221> misc_feature
<222> (891)..(897)
<223> primer linker

<400> 4
ccatggctgc tgcactgtcc cccctctccc agcgctttga gcgcatcgcg gtccagccgc 60

tgaccggcgt cctgggcgcc gagatcaccg gcgtcgacct gcgcgagccg ctcgacgaca 120

gcacctggaa cgaaatcctc gacgcgttcc acacttacca ggtcatctat tttcccggcc 180

aggcgatcac caacgaacag cacatcgcct tcagccggcg cttcggcccc gtcgatcccg 240

tgcccctgct caagagcatc gaagggtatc cagaggtgca gatgatccgc cgcgaagcca 300

acgaaagcgg gcgtgtgatc ggtgatgact ggcacaccga cagcaccttc ctggacgcac 360

cgccggccgc cgtggtgatg cgcgcgatcg acgtgcccga gcatggcggc gacaccggtt 420

ttctgagcat gtacaccgcg tgggagacgc tgtcgcccac catgcaggcc accatcgaag 480

ggttgaacgt agtgcacagc gccacgcgtg tgttcggctc gctctaccag gcccagaacc 540

ggcgcttcag caacaccagc gtcaaggtga tggacgtcga cgcgggcgac cgtgaaaccg 600

tgcacccccc ggtggtgacc catccgggca gcggctgcaa gggcctgtac gtgaaccagg 660

tctattgcca gcgcatcgag ggcatgaccg atgccgaaag caaaccgctg ctgcagttcc 720

tgtacgagca tgcgacacgg ttcgatttca cctgccgcgt gcgctggaag aaggaccagg 780

tcctggtctg ggacaacctg tgcacgatgc accgggccgt acccgactac gcgggcaagt 840

tccgctacct gacgcgcacc acggtcggtg gcgtgcgccc ggcgcgctag tgagctc 897

<210> 5
<211> 919
<212> DNA
<213> Artificial Sequence

<220>
<223> AAD-1 (v3) primary sequence

<220>
<221> misc_feature
<222> (1)..(2)
<223> primer linker

<220>
<221> misc_feature
<222> (6)..(8)
<223> additional alanine codon (GCT)

<220>
<221> misc_feature
<222> (894)..(919)
<223> primer linker

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cactcactgg tgtccttggt gctgagatca ctggagtgga cttgagggaa ccacttgatg 120

acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcatc tactttcctg 180

gccaagcaat caccaatgag cagcacattg cattctcaag aaggtttgga ccagttgatc 240

cagtgcctct tctcaagagc attgaaggct atccagaggt tcagatgatc cgcagagaag 300

ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttccttgatg 360

cacctccagc tgctgttgtg atgagggcca tagatgttcc tgagcatggc ggagacactg 420

ggttcctttc aatgtacaca gcttgggaga ccttgtctcc aaccatgcaa gccaccatcg 480

aagggctcaa cgttgtgcac tctgccacac gtgtgttcgg ttccctctac caagcacaga 540

accgtcgctt cagcaacacc tcagtcaagg tgatggatgt tgatgctggt gacagagaga 600

cagtccatcc cttggttgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc 660

aagtctactg tcagagaatt gagggcatga cagatgcaga atcaaagcca ttgcttcagt 720

tcctctatga gcatgccacc agatttgact tcacttgccg tgtgaggtgg aagaaagacc 780

aagtccttgt ctgggacaac ttgtgcacca tgcaccgtgc tgttcctgac tatgctggca 840

agttcagata cttgactcgc accacagttg gtggagttag gcctgcccgc tgagtagtta 900

gcttaatcac ctagagctc 919

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> rdpA(ncoI) 5' primer

<400> 6
cccatggctg ctgcactgtc ccccctctcc 30

<210> 7
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> 3'saci primer

<400> 7
gagctcacta gcgcgccggg cgcacgccac cga 33

<210> 8
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> BstEII/ Del NotI 5' primer

<400> 8
tggtggtgac ccatccgggc agcggctgca agggcc 36

<210> 9
<211> 295
<212> PRT
<213> Sphingobium herbicidovorans

<400> 9

Met His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1 5 10 15

```
Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
            20                  25                  30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60

Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65                  70                  75                  80

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255
```

```
Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295
```

<210> 10
<211> 295
<212> PRT
<213> Artificial Sequence

<220>
<223> AAD-1 v2 Translation

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> Different from v1

<220>
<221> MISC_FEATURE
<222> (212)..(212)
<223> Different from v1

<400> 10

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Met Ala Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1               5                   10                  15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
            20                  25                  30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60

Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65                  70                  75                  80

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110
```

```
Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295
```

<210> 11
<211> 296
<212> PRT
<213> Artificial Sequence

<220>
<223> AAD-1 v3 Translation

<220>
<221> MISC_FEATURE
<222> (2)..(3)
<223> Different from V1

<400> 11

```
Met Ala His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile
1               5                  10                  15

Ala Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val
            20                  25                  30

Asp Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp
        35                  40                  45

Ala Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr
    50                  55                  60

Asn Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro
65                  70                  75                  80

Val Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile
                85                  90                  95

Arg Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His
            100                 105                 110

Thr Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg
        115                 120                 125

Ala Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met
    130                 135                 140

Tyr Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu
145                 150                 155                 160

Gly Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr
                165                 170                 175

Gln Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp
            180                 185                 190

Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
        195                 200                 205

Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
    210                 215                 220

Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
225                 230                 235                 240
```

Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
245 250 255

Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
260 265 270

Ala Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr
275 280 285

Val Gly Gly Val Arg Pro Ala Arg
290 295

<210> 12
<211> 888
<212> DNA
<213> Bradyrhizobium japonicum USDA 110

<400> 12
atgacgatcg ccatccggca gcttcagacg cattttgtcg gccaggtttc cggcctcgat 60

ttgcgaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac 120

gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg 180

aacttcggcc agcgcgagga cgcgcgcggc ggcacggtca ccaaggagaa ggactaccgg 240

ctgcaatccg gcctgaacga cgtctccaat ctcggcaagg acggcaagcc gctggccaag 300

gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc 360

cgtcccattc ccgcaaaatt ctcgctgctg tcggcgcgcg tggtgaaccc gacgggcggc 420

aacaccgaat tcgcggacat gcgcgccgcc tatgacgcgc tcgacgacga gaccaaggcc 480

gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc 540

accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc 600

acccatccgg tccaccgccg caagtcgctg tatctctcgt cgcatgccgg caagatcgcc 660

agcatgagcg tgccggaggg gcggctgctg ttgcgcgatc tcaacgagca cgcgacgcag 720

ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc 780

cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgccgcgcg 840

acggtggcgg ggacggagcc gacggtgcag cagcaggcgg cggagtag 888

<210> 13
<211> 289
<212> PRT
<213> Bradyrhizobium japonicum USDA 110

<400> 13

```
Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
1               5                  10                  15

Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
            20                  25                  30

Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
        35                  40                  45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
    50                  55                  60

Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
        115                 120                 125

Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
    130                 135                 140

Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
145                 150                 155                 160

Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
                165                 170                 175

Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
        195                 200                 205

Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
    210                 215                 220

Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240
```

Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
245 250 255

Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
260 265 270

Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
275 280 285

Val

<210> 14
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> brjap 5'(speI)

<400> 14
actagtaaca aagaaggaga tataccatga cgat 34

<210> 15
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> br jap 3' (xhoI)

<400> 15
ttctcgagct atcactccgc cgcctgctgc tgc 33

<210> 16
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 forward primer

<400> 16
gtaaaacgac ggccagt 17

<210> 17
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 reverse primer

<400> 17

caggaaacag ctatgac 17

<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> NcoI of Brady

<400> 18
tataccacat gtcgatcgcc atccggcagc tt 32

<210> 19
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> SacI of Brady

<400> 19
gagctcctat cactccgccg cctgctgctg cac 33

<210> 20
<211> 525
<212> PRT
<213> Glycine max

<400> 20

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1 5 10 15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
20 25 30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
35 40 45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
50 55 60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65 70 75 80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
85 90 95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
100 105 110

```
Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350
```

```
Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525
```

<210> 21
<211> 525
<212> PRT
<213> Artificial sequence

<220>
<223> Doubly mutated Soybean EPSPS protein: threonine 183 converted to isoleucine; proline 187 converted to serine

<400> 21

```
Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1               5                   10                  15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
            20                  25                  30
```

```
Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
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Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
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Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
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Molecular biology of weed control *

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Key words: deleterious transposons, herbicide-resistant crops, introgression, transgenetic mitigation, weed control

Abstract

The vast commercial effort to utilize chemical and molecular tools to solve weed control problems has had a major impact on the basic biological sciences as well as benefits to agriculture, and the first generation of transgenic products has been successful, while somewhat crude. More sophisticated products are envisaged and expected. Biotechnologically-derived herbicide-resistant crops have been a considerable benefit, yet in some cases there is a risk that the same useful transgenes may introgress into related weeds, specifically the weeds that are hardest to control without such transgenic crops. Biotechnology can also be used to mitigate the risks. Molecular tools should be considered for weed control without the use of, or with less chemicals, whether by enhancing crop competitiveness with weeds for light, nutrients and water, or via allelochemicals. Biocontrol agents may become more effective as well as more safe when rendered hypervirulent yet non-spreading by biotechnology. There might be ways to disperse deleterious transposons throughout weed populations, obviating the need to modify the crops.

Abbreviations: ACCase – acetyl CoA carboxylase; ALS – acetolactate synthase; BD-HRC – biotechnologically-derived herbicide-resistant crops; DT – deleterious transposons; EPSP synthase – enolpyruvate-shikimate phosphate synthase; TM – transgenetic mitigation.

Introduction

It may seem hard to realize that there has been and is an intimate, bidirectional relationship between molecular biology – a most basic biological science – and weed control, the most base as well as basic agricultural practice. Weed control, as practiced for millennia is base because it has employed the majority of mankind on this planet since crops were domesticated, yet has been the lowest paying mass employment known. Weed control can be one of the most physically taxing tasks, yet typically more women are employed in weeding than men. Without weed control there is virtually no crop to harvest. The advent of mechanization (tractor plowing and cultivating) replaced much of the hand labor in the developed world as well as the developing parts of the third world. Mechanical weed control is fraught with high energy costs, facilitates soil erosion and compaction and has been largely replaced by chemical weed control using herbicides that can selectively eradicate weeds from crops. As countries industrialize and develop economically, cheap farm labor becomes unavailable, increasing the necessity for cost-effective chemical weed control.

Selectivity between a crop and its associated weeds is no mundane trait to find in a chemical. It is based on the immense biodiversity of metabolic pathways abounding in the plant kingdom. Too often there is no selective chemical that can control a particular weed in a particular crop, and the weed infestations understandably worsen based on simple ecological, genetic and biochemical principles. No single agricultural practice used extensively in the past to control weeds has remained as useful as initially presumed.

* International Standards Organization (ISO) accepted common names of herbicides are used throughout. The chemical names can be found in Tomlin (1994). Occasionally, molecular biologists have used non-accepted common names for herbicides. Their synonyms will be given in parenthesis at first use in this review.

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After one weed problem is solved, there are other weed species that will fill the ecological vacuum left behind. Thus, weeds have either evolved resistance to most commonly used herbicides, or weed species that had never been controlled by the particular herbicide replace the weed species controlled. This is most apparent in monoculture where weeds closely related to crops are becoming the greatest problems; grass weeds in grain crops; legumes in soybeans, brassicas in oilseed rape, etc. As most selectivities between crop and weed are due to catabolic degradation of the herbicide by the crop, closely related weeds are to be expected to have similar catabolic pathways as the crop. This is one major reason that biotechnologically-derived herbicide-resistant crops (BD-HRC) have become so useful, and that biotechnology has been utilized to produce such crops as well as to find new herbicide targets (see excellent recent review by Cole & Rodgers, 2000). Selectivity is enhanced by inserting exogenous resistance genes into the crops or by selecting natural mutations. This also leads to one major concern about transgenic HRCs, that the transgene will genetically introgress into related weeds.

These and other issues will be reviewed below, as well as a call for new concepts of using molecular biology for weed control that do not necessarily utilize HRC, with some possible examples for consideration. Indeed, it is highly unfortunate that a recent World Bank report (Kendall et al., 1997) dealing with how molecular biology can alleviate constraints to world food production has only delineated insects, pathogens and lack of water as the major constraints where molecular biology can assist. This report ignored weeds, even though the greatest variable input into agriculture is the cost of weed control, whether (fe)manual, mechanical or chemical, with or without transgenics. And there are many unsolved weed problems lowering yields over vast areas of the world. If the weeds could be controlled, crop yields would increase without additional inputs.

Contributions of weed science to biochemistry and molecular biology

It was said above that there has been a bidirectional relationship often not realized between weed control and molecular biology and biochemistry. Most believe that the relationship has been unidirectional from biochemistry and molecular biology to the farmer. This is hardly so. The dire need for developing cost effective chemical weed control systems has lead to a vast industrial investment to find and develop selective herbicides and later BD-HRC. Virtually all herbicides marketed are the result of random screening of chemicals to obtain active leads, as the result of huge efforts of procurement and chemical synthesis. Once a lead has been obtained, further syntheses around it are used to find compounds with greater activity and then selectivity. After such compounds have been found and marketed, they opportunistically become widely used tools of the physiologists and biochemists, first to find a site of action and then as 'antimetabolites' to further understand and modulate metabolic pathways. Thus the advent of 2,4-D assisted in understanding auxin action, atrazine and diuron (DCMU) in understanding photosystem II, paraquat for photosystem I, dinitroanilines in dealing with tubulin assembly into microtubules, dichlobenil for cellulose biosynthesis, etc. Herbicides are the antimetabolites of choice in dealing with key enzymes such as glutamine synthase [glufosinate (phosphinothricin)], acetolactate synthase (ALS) (many herbicides), acetyl-CoA carboxylase (ACCase) (many herbicides), dihydropteroate synthase (asulam), enolpyruvate-shikimate phosphate synthase (EPSP) (glyphosate), and phytoene desaturase (many herbicides). We know far more about the enzymes blocked by herbicides than most other enzymes in plants. The genes for most of these enzymes have been isolated and used in transgenic programs. Such research transcended plant biochemistry and agriculture. For example, it was discovered through comparative genomics that plant and trypanosome β-tubulins were similar to each other and different from mammalian β-tubulin. The dinitroaniline herbicides then proved to be excellent trypanocides (Chan et al., 1993; Bell, 1998).

The repetitive (mis)use of single herbicides in monoculture over many years predictably lead to the evolution of herbicide-resistant weeds (Gressel & Segel, 1978; Powles & Holtum, 1994). The advent of triazine resistance was crucial to the understanding of the role of the *psb*A gene product in the photosystem II binding site, leading to innumerable studies of photosynthesis, biophysics and biochemistry correlated with molecular structure of the gene product. Again this transcended plant molecular biology and weeds when, based on this agricultural phenomenon, similar mutations were generated in photosynthetic bacteria. The mutant and natural *psb*A gene products were crystallized and analyzed, leading to new insights into 'drug' (ligand) binding and design, as well as a No-

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bel Prize in medicine (Michel & Deisenhofer, 1988; Deisenhofer & Michel, 1989).

Information from herbicide resistance provided the theoretical underpinning for designing transient drought resistant plants. Harvey and Harper (1982) first promoted the idea that paraquat resistance can be similar to oxidative stress tolerance. This was later extrapolated to being similar to transient drought tolerance (Malan et al., 1990). This has allowed developing quick pre-tests with paraquat to ascertain the level of transient drought tolerance of transgenic plants bearing genes designed to confer oxidative stress resistance.

Genes coding for herbicide resistance developed for agriculture became the selectable markers of choice for generating transgenics, supplanting antibiotic resistance, even when there was no plan for registering the herbicide for use in that crop.

The huge corporate investment in HRC and *Bacillus thuringiensis* (Bt) toxin containing crops due to perceived market size, resulted in the gain of much of our knowledge on promoters, organelle-specific and transit peptides, as well as more recently in organelle transformation. This corporate investment in basic plant molecular biology was manifold greater than the public sector effort, and the spill-over was great. Let us remember that it was market-driven transgenic research, and the market is for weed control.

Needs for transgenics not being met

As much of the basic and applied effort, is market-driven, the predominant weed problems being solved are those where corporations perceive maximal gain. Thus, the parasitic flowering *Striga* spp. that are claimed to decimate half the maize, sorghum and legume yields of 100 million farmers in Africa are not considered a market. This is despite the demonstration that genes already available allow selective control of these and similar parasites on transgenic crops (Joel et al., 1995). The genes are not even being made available by the multinationals to public-sector researchers for crossing or insertion into African crop varieties (Anonymous, 1999b).

There is a dire need for HR wheat and rice to enable the control of grass weeds that have evolved resistance to most graminicides (grass-killing herbicides) that can be used in these two crops. As these crops are predominantly cultivated with farmer-saved seed, especially in marginal areas, the commercial gain from seed sales does not attract the multi-nationals. Again, many of the genes that could be used are already available, and could be cheaply inserted. The cost of isolating genes and producing constructs that allow normal yields, as well as ascertaining toxicological safety have already been performed. Introducing the same constructs in other crops is far less costly, but the corporate reluctance remains.

Predominantly hand labor and mechanical methods are still used for controlling weeds in vegetable crops, even in developed countries due to a paucity of registered selective herbicides. Such crops are especially adaptable to a transgenic approach using existing genes and herbicides. A large premium could be added to seed price when weed control costs are lowered, but the small market size and minuscule additional herbicide sales have hampered the registration of appropriate herbicides and have not enticed the development of such crops.

Uses of molecular biology in weed control

Not all applications of molecular biology are commercial. For example, there is a necessity to be able to taxonomically classify weeds, as there are differences in selective control among related weeds, as epitomized with weeds of the genus *Amaranthus* (Mayo et al., 1995). Molecular taxonomy has been of great assistance and has often provided the decisive data in many cases on whether two similar *Amaranthus* species were actually one, or were separate, or were hybrids, depriving classical taxonomists of their endless battles. This has been done recently with the taxonomically-challenging *Amaranthus* spp., which also have a propensity to cross among themselves (Lanoue et al., 1996; Chan & Sun, 1997; Wetzel et al., 1999a). Knowing relatedness will be required for assessing the risks of crop gene introgression into weeds, and has been used to trace whether an *Amaranthus* biotype evolved resistance by introgression, vs. by its own internal mutation (Wetzel et al., 1999b).

Some weeds are difficult to classify, especially the differentiation of some wild *Oryza* species from the feral forms of cultivated rice (*Oryza sativa*). This is very important to know, as the wild species do not introgress freely with rice and the feral form does (Morishima, 1984; Ling-Hwa & Morishina, 1997; Mariam et al., 1996; Maesako et al., 1995; Majumder et al., 1997; Cohen et al., 1999). One must know which *Oryza* species are in a given area for

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risk assessment of transgenic rice, yet little is known about which species predominate and where (Baltazar & Janiya, 1999). Indeed, the classical taxonomy is so complicated that it was found using molecular techniques that many accessions of wild rice species in the collection of the International Rice Research Institute (IRRI) were mis-classified (Martin et al., 1997).

Most of the present uses of molecular biology are to find new herbicide targets and to generate HRC.

Genomics to elucidate herbicide targets

The use of genomics to elucidate pharmaceutical targets and to develop rapid screens for chemicals affecting those targets has spread to the pesticide industry with a vengeance (Berg et al., 1999). This is despite the lack of proven efficacy of such techniques in drug or pesticide design so far, as well as the high cost. This effort is motivated by the perceived potential of patenting possible future targets for herbicide discovery.

Indeed, the calibrational use of antisense and knockout techniques with known herbicide targets does yield inhibited or dead plants (Höfgen et al., 1994, 1995; Haake et al., 1998). Because of the redundancy within and among gene families, as well as alternative pathways for many processes, many enzymes are not suitable targets for herbicide action. Additionally, it is not realized by many that it is not usually sufficient to inhibit a primary non-redundant pathway that has no alternative routes. Most known herbicides rarely completely inhibit a pathway, and plants rarely die by starvation. A potato or bean plant will live for weeks in the dark, but inhibitors of photosystem II or I kill the same plants in days or hours (respectively) in the light, yet these inhibitors do not completely block these processes. The blockages imposed by these herbicides typically cause the accumulation of toxic products or weaken the weeds such that other factors kill plants. The toxicity of such blockages cannot be predicted based on the knowledge of enzyme inhibition alone. For this reason, the results of incomplete suppression of gene expression achieved by antisense or overexpressive co-suppression have an advantage over the knock out or deletion of genes for elucidating potential targets. Once such potential targets are pinpointed, simple, inexpensive, miniaturized, high throughput rapid screens are developed for assaying new compounds generated by combinatorial and conventional chemistry.

The drawbacks of Arabidopsis *genomics*

The pronouncements by the companies promoting genomics for elucidating potential herbicide targets state that this is being performed in *Arabidopsis thaliana* cv. Columbia. This might seem an obvious choice because of the almost fully sequenced *Arabidopsis* genome, but is it the best choice? At least one company searching for potential fungicide and insecticide targets is not using yeast and *Drosophila*, respectively, despite the extensive genome sequencing of these species. The reasoning is that these two sequenced species are not pathogens or pests of crops and thus do not bear many of the requisite pest traits that would be targets for intervention by new pesticides. They are searching for their novel pesticide targets in agricultural pathogens /insect pests. Despite Arabidoptologists terming their pet a 'weed', it is not. *Arabidopsis* spp. lacks the complex of traits that defines an agricultural weed; i.e. it cannot and does not exist in agro-ecosystems, and cannot compete with crop plants and does not reduce crop yields. The Columbia strain used has become a domesticated species due to greenhouse cultivation. Wild traits such as secondary dormancy of seeds have been lost from the laboratory strain; researchers had to revert to wild strains to find genes controlling this trait (Steber et al., 1997). The genes conferring disease resistance to a disease naturally found in the wild are physically deleted from the genome of the lab strain (Stahl et al., 1999). The disease resistance genes previously isolated from laboratory *Arabidopsis* strains confer resistance to artificial pathogens, i.e. not ones naturally attacking. One might posit that this minute wild species uses a growth strategy to compete in its own ecological niche that is very different from the strategy used by most cultivated as well as weedy species. Minimizing small genome size by deleting unused genes may well be an evolutionary strategy for efficiently completing a rapid life cycle with a minimum of costly DNA synthesis in the ecological niche of *Arabidopsis*. Slower growing, longer life cycle, more competitive weed species may be able to afford the luxury of large gene families, an abundancy of redundancies and non-coding regions; i.e. the typical DNA strategy of most species. Some potential herbicide targets may be found in *Arabidopsis thaliana* cv. Columbia that are without large families or other redundancies in other species. *Arabidopsis* genomics can assist in identifying target sites in the most primary of pathways, but one might question whether this is what is most needed. The worst of the Worlds Worst Weeds (Holm et al., 1977) are the

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perennial *Cyperus* (nutsedge) species. These are hard to control with herbicides affecting primary pathways, presumably because *Cyperus* contains an abundance of vegetative storage proteins that allow them to come back, phoenix like, if a herbicide does kill leaves. Studying the genomics of *Cyperus* vegetative storage proteins may lead to finding a good cypericide, or an additive to a herbicide that will prevent *Cyperus* regrowth. It is doubted that minimalist *Arabidopsis* will contain such genes. The need for graminicides (grass-killing herbicides) is especially acute, especially in grain crops because of the evolution of resistance to the presently available products. Will *Arabidopsis* be of help? The presently leading graminicides do not affect *Arabidopsis* – they act by inhibiting a grass-specific ACCase that is not found in *Arabidopsis* or other dicots. Considering history and biodiversity, is it not much to expect that the leads for the next generation of graminicides will come from *Arabidopsis*? Thus, the dependency on *Arabidopsis* genomics may well be a misdirected paradigm for finding the best herbicide targets to fit the major needs of agriculture. Indeed the chauvanism of many Arabidopsologists is reminiscent of those suggesting that all elephant genes would be found in *E. coli.*

The success of biotechnologically-derived herbicide resistant crops (BD-HRC)

Millions of hectares are being planted with BD-HRC, with insect resistance in second place, with both traits often 'stacked' in the same seeds to enhance their value. Farmers perceive a utility in BD-HRC, as they have many alternatives and still repeatedly purchase seed of BD-HRC. The real values of BD-HRC come from instances where there really are no viable weed control methods (e.g. due to evolved herbicide resistances in weeds), and the impact that such BD-HRC could lead to a more sustainable world food production. BD-HRC are the subject of a recent book (Duke, 1996), as well as an in depth review of the molecular aspects (Cole & Rodgers, 2000).

The easiest way to obtain selectivity among closely related species is to engineer resistance to a general herbicide into the crop. For example, it has already been shown that red rice (*Oryza sativa*) is easily controlled by glufosinate, in transgenic rice (Sankula et al., 1997a,b) bearing the *bar* gene that confers resistance to this herbicide (Oard et al., 1996). The immediate answer to multiple resistance problems in weeds of wheat in major growing areas is to engineer resistances to inexpensive herbicides (Gressel, 1988). Neither the chemical nor the biotechnological industries have shown particular interest in generating BD-HRC in wheat and rice. As too little profit is perceived to come from wheat or rice seed or even from generic herbicides, it may be necessary to have wheat and rice engineered by the public sector. Glufosinate resistance has been engineered into wheat, more as a marker gene than for agronomic utility (Weeks et al., 1993). A request has been made for field use of glufosinate resistant-rice in the USA (Anonymous, 1999), which has been allowed, despite the fact that it is well known that the gene can introgress into con-specific red rice (Sankula et al., 1998).

BD-HR wheats and rices may be an answer to the major problems of these crops. Insert a gene into wheat or rice conferring resistance to a broad spectrum herbicide and you can control weeds that evolved resistance in wheat and even closely-related grasses, including red, weedy, and other wild rices in rice (Gressel, 1999a,b,c). The transgenes will allow problems of resistance that have evolved to be overcome especially, the problems of cross-resistances (where one evolutionary step confers resistance to a variety of chemicals) and multiple resistance (where a sequence of evolutionary steps with different selectors, confers resistance to a variety of chemicals). The use of non-plant transgenes may also allow farmers to overcome the natural resistances in weeds closely related to the crop. One can even choose bacterial genes for inexpensive long-established herbicides such as dalapon (Buchanan-Wollaston et al., 1992), modify them for plant codon usage, and introduce them into grain crops (Gressel, et al., 1997a). Such genes have the advantage that it is harder, albeit not impossible, for the weeds to mimic bacteria than to mimic plants in evolving resistance.

The problems of interclass cross resistances and multiple resistances in wheat have necessitated considering the generation of BD-HR oilseed rape, especially in Australia (Gressel, 1999b). Oilseed rape has become an excellent rotational crop alternating with wheat in many places where wheat is grown. There are many agronomic advantages to rotating a dicot with a monocot, especially vis a vis weeds. It should be far easier to eliminate grass weeds in oilseed rape than in wheat, as there are more selective graminicides available for use in dicot crops. This is usually correct except in Australia, where *Lolium rigidum* has successfully evolved widespread target site and intergroup cross resistances as well as multiple resistances to all

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these graminicides (though not in all fields). It is additionally necessary to generate BD-HR oilseed rape (*Brassica napus*) to allow control of closely related *Brassica* weeds including those such as *Raphanus raphanistrum*. Here we can make the case for a need for management strategies with BD-HRC as with any selective herbicide. Atrazine-resistant BD-HR oilseed rape was widely and exclusively used to control *R. raphanistrum* in Australia. It was generated by backcrossing the gene in from a mutant *B. campestris* that evolved in Canada (Souza-Machado, 1982). Inevitably, there are reports that this weed has evolved resistance to triazines (Cheam et al., 2000), as well as resistance to ALS-inhibitors (Cheam et al., 1999) and to diflufenican (Cheam et al., 2000). Thus, one must engineer resistances into oilseed rape that allows control of both *Lolium rigidum* and *Raphanus raphanistrum,* with all their resistances. To delay further resistances to the few remaining herbicides, perhaps gene stacking and using herbicide mixtures is of necessity (see below).

Only the first generations of BD-HCR have been released, and considerable improvements are to be expected. There are far too few concrete molecular and biochemical data published about the properties of these crops and thus there are problems in evaluating their properties to allow suggestions for improvements. Thus, some of what will be said below should be considered as speculative. Two types of gene have been used to generate herbicide-resistant crops: (1) where the gene product detoxifies the herbicide; (2) where the herbicide target has been modified such that it no longer binds the herbicide. One could envisage other types such as exclusionary mechanisms, sequestration, etc., but they have yet to be found and thus not utilized.

The resulting BD-HRC with each type of resistance are rather different and thus will be discussed separately.

Metabolically-resistant BD-HRC —

Many crops bearing transgenes coding for highly specific enzymes that metabolically catabolize herbicides have been generated (Cole & Rodgers, 2000). These include for example, bromoxynil resistance crops bearing a nitrilase (Freyssinet et al., 1996; Stalker et al., 1996), glufosinate-resistant crops bearing an acetyl-transferase (cf. Vasil, 1996), 2,4-D resisting crops bearing a highly specific soluble cytochrome P-450 monooxygenase (Streber & Willmitzer, 1989), phenmidipham resisting crops bearing a bacterial gene (Streber et al., 1994), and dalapon resisting crops bearing a dehalogenase (Buchanon-Wallaston et al., 1992). Of these, only the bromoxynil- and glufosinate-resistant crops have reached commercialization. All the commercial genes used commercially are of bacterial/actinomycete origin, despite the fact that plants contain genes for herbicide resistance, which is the basis for most natural metabolic selectivities used for 45 years, yet plant genes conferring metabolic resistance have not been used commercially as yet. There are recent reports using non-prokaryotic genes to confer resistance, but none are yet commercial, and whether they confer sufficient resistance is not clear. A rabbit esterase gene confered resistance to thiazopyr via degradation (Feng et al., 1998). The expression of plant and animal P_{450} transgenes confered phenylurea resistance (Inui et al., 1999; Siminsky et al., 1999). Transgenes encoding maize glutathione transferases increased the level of herbicide resistance (Jepson et al., 1997).

Unlike the target site resistances that are fraught with problems (see below), the crops generated with metabolic resistances seem to be problem-free, with little metabolic load conferred by generating the small amount of enzyme needed. The toxicology is simplified because the transgene product typically initiates a cascade of events whereby the herbicide eventually disappears.

There has been an assumption that one cannot use catabolic enzymes to confer resistance to fast-acting herbicides. This view is of doubtful relevance as biotechnologists should be able to perform as well as plants. Inhibitors of protoporphyrinogen IX oxidase (protox), which actually cause the accumulation of the photodynamically-toxic product (Böger & Wakabayashi, 1999) induce photodynamic death of plants within 4–6 h in bright sunlight. Beans are immune to some members of this group, e.g. acifluorfen, because they possess a specific homoglutathione transferase and contain enough homoglutathione to stoichiometrically degrade these herbicides before they can damage the crop (Skipsey et al., 1997). Similarly, strains of *Conyza bonariensis* contain a complex of enzymes capable of detoxifying the reactive oxygen species generated by the photosystem I blocker paraquat, and keeping the plants alive until the paraquat is dissipated (Ye et al., 2000; Ye & Gressel, 2000).

As almost all herbicides are either degraded in the soil or in some plant species, one should be able to find more genes for catabolic resistance to those herbicides and then be able to rapidly generate herbicide-resistant

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crops with metabolic resistance than with target site resistance.

Target-site resistant crops

Biotechnology has been used to generate target site HRC crops by two means – transfer of field or laboratory generated mutants into crop varieties by genetic means; or to do the same transgenically.

One must assume a fitness penalty with any target site HRC. This is simply due to the fact that if the resistant mutation was neutral or very near neutral there would be naturally-resistant populations pre-existing in the wild due to Sewall Wright drift. As all the mutations were found at a low frequency; one in a million or less, the resistant traits are not of near neutral fitness. This was most obvious with triazine resistance, which was crossed from weedy populations of *Brassica campestris* (=*B. rapa*) that evolved resistance, into oilseed rape (*B. napa)* (Souza-Machado, 1982). This *psb*A gly 264 ser mutation is plastid inherited, allowing researchers to test fitness in the near isonuclear eighth back cross (Gressel & Ben Sinai, 1985), and in isonuclear reciprocal hybrids (Beversdorf et al., 1988). There was a consistent 20% productivity loss when the crop was grown alone and a much greater fitness loss when the resistant biotypes were grown in competition with the sensitive biotype. A 20% yield loss does not mean that such crops are without value. This triazine-resistant oilseed rape is widely grown in Australia because some pernicious weeds can be inexpensively controlled. Still, the triazine-resistant varieties will probably eventually be supplanted by transgenics, which do not have the fitness penalty.

Despite a plethora of papers claiming no fitness penalty in acetolactate synthase mutant and transgenic plants (see Saari et al., 1994), elegant experiments by Bergelson et al. (1996) with transgenic *Arabidopsis* did indeed show that such a penalty exists. Previous competitive fitness experiments either started with transplants and/or did not measure seed output. The two most competitive phases in a plant's life cycle are at establishment, when large populations of seedlings self-thin to a single plant per parent, and fertilization, when thousands of pollen grains compete on stigma and through style. If competition is not measured seed to seed, then the fitness penalty is unknown.

The natural resistance mutations to ALS and ACCase derived from maize tissue cultures, or via pollen mutagenesis and then backcrossed into inbreds seem to have produced normally yielding hybrids. There have been more problems with the transgenics than the mutants, as successful as they have been in the market place. The magnitude of resistance with transgenics is not as high as it is with the natural mutations, both with ALS and EPSP-synthase target site resistances. The first single-site mutant pro101ser EPSP-synthase transgenic plants tested were insufficiently resistant to glyphosate for field use. Thus, considerable effort was made to increase the level of resistance and repair the huge shift in K_m towards natural substrates by adding both second site directed mutations as well as enhanced promoters (Padgette et al., 1996; Lebrun et al., 1997). The necessity to do this was the basis for the claimed invincibility of glyphosate-resistant crops from evolved resistance in weeds; it would take too many simultaneous mutations for resistance to evolve (Bradshaw et al., 1997). This thesis was published after there was already known natural metabolic resistance in legumes (Komossa et al., 1992) as well as natural resistance due to enhanced levels of the target enzyme in *Lotus corniculatus* (Boerboom et al., 1990). These flaws in the invincibility theory were pointed out by Dyer (1994) and reiterated and updated by Gressel (1996). The only difference that could be found between one of the recently evolved glyphosate-resistant *Lolium rigidum* populations having a seven-fold increase in the I_{50} value, and the wild type was the presence of a doubled level of EPSP-synthase (Gruys et al., 1999). *Lotus corniculatus* with a doubled level of EPSP-synthase also had a seven-fold increase in glyphosate resistance (Boerboom et al., 1990). This is not the only case where it has been possible to confer herbicide resistance by over-expression of the gene encoding its target site. Glufosinate resistance was achieved in tissue culture by this means with overproduction of glutamine synthase (Donn et al., 1984; Deak et al., 1988). Similarly, poplars were transformed for elevated glutamine synthase to enhance nitrogen utilization (Gallardo et al., 1999). The transgenic poplars are more resistant to glufosinate than the wild type (Francisco M. Cánovas, pers. comm.). Additionally, transgenic tobacco plants overexpressing protoporphyrinogen IX oxidase fivefold in chloroplasts are resistant to a discriminatory dose of 20 μM acifluorfen, which severly inhibited the wild type (Lermontova & Grimm, 2000). Unfortunately, in this latter case, there are no quantitave data on the magnitude of resistance.

The nail in the coffin of invincibility came with a recent identification of glyphosate- resistant *Eleucine indica* in Malaysia (Lee & Ngim, 2000). This

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material was resistant to far greater than typical field application rates of glyphosate. It was recently disclosed that this material bore a target-site resistance to glyphosate (Doll, 2000). The single mutation in the binding domain of this material was at a position equivalent to the pro101ser in the *Salmonella typhimurium aroA* resistance gene (G. Dill, pers. comm.), the same position that was regarded as insufficiently resistant in trangenic resistance genes (Padgette et al., 1996).

In retrospect, there is good reason to expect that transgenics will be less resistant than target site mutants bearing the same transversion. Recurrent selection of a natural mutant will select for homozygous resistant individuals. This cannot happen with the present generation of transgenics. They bear and express both the native and transgenic enzymes (although the ratios of the two have never been published to the best of my knowledge). Thus, transgenic plants with target site resistance are functionally heterozygous and will remain so despite recurrent selection. Homozygosity can only be achieved at present when totally exogenous genes such as those for metabolic resistance are used, as they do not compete with native genes. When the herbicide is applied, target site HRC must depend on the transgenic derived enzyme while the native is inhibited, perhaps even causing phytotoxic precursors to accumulate.

There have been continuing problems with some glyphosate-resistant crops, suggesting that the critical balance of transgenic and native enzymes is not optimal. Initial varieties of maize, about to be released four years ago were retracted because late season herbicide application caused pollen sterility. Similar problems of obtaining vegetative (only) glyphosate-resistant cotton plants continue to plague cotton growers. The period of pollen production is well known to be subject to inhibition by minor, chemical, metabolic or environmental perturbations. There have been recent reports of stem brittleness and cracking in untreated glyphosate resistant soybean (Anonymous, 2000) suggesting an overproduction of product leading to increased lignin formation when both the native and transgene derived enzymes are operative.

One does not know yet whether such glyphosate-resistant transgenic plants are transiently weakened until the herbicide is dissipated. This period after glyphosate application is analogous to sub-lethal treatments of glyphosate to non-transgenic plants. When legumes are given sub-lethal applications of glyphosate they are unable to produce phytoalexins after elicitation, and are far more susceptible to disease (Keen et al., 1992; Sharon et al., 1992). A recent study purporting to ascertain whether phytoalexin biosynthesis was compromised after glyphosate treatment of glyphosate-resistant soybeans (Lee et al., 2000) was artifactual. The authors measured only constitutive levels of glyceollin, without elicitation, and their conclusion vis a vis phytoalexins, which are by definition elicited, are thus without basis.

There at least two apparent solutions to the problems arising from the functional heterozygote status of transgenic target site resistant plants. (1) One way is to try to enhance the metabolism of the herbicide, obviating the problem. This may have been done using the *gox* gene coding for an enzyme degrading glyphosate together with target-site resistance (Mannerlöf et al., 1997). There are no data indicating how quickly the transformants degraded the glyphosate in this case. (2) A better solution might be to begin with a 'clean slate'. Target-site resistant plants should be generated via replacement, i.e. in plants that have had the native gene deleted or functionally irreversibly 'knocked out'. This can be done by classical deletion mutagenesis with UV or gamma irradiation, but this causes multiple lesions, and the screening for particular mutants is not easy. Molecular techniques show far more promise as they are more 'surgical', i.e. there is less likelihood of multiple deletions along a chromosome, leading to complications from linked deletions. These techniques include T-DNA tagging (Zupan & Zambryski, 1995; Choe & Feldmann, 1998) and transposon mutagenesis (Tissier et al., 1999). There are an estimated 3000 genes that might be target sites of herbicides (Berg et al., 1999), giving mutations that will have to be rescued by the specific gene products of interest as the selectable markers. The most elegant new technique is with specific viral vectors spliced to the gene to be silenced. After cloning into the crop, some black magic mechanism of virus-induced gene silencing turns off both the viral and the endogenous gene (Baulcombe, 1999). At least one of the virus vectors used is transmitted through seeds and by pollen, so following generations remain with the gene suppressed (Lister & Murant, 1967), but if the gene is not ever re-conducted is not certain.

After the native gene is suppressed, then it should be possible to obtain transgenics with various levels of transgene expression in functional homozygotes, and choose those with the optimal expression level for agricultural release. The gene used may have to be very different in sequence from the native gene, as

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viral-induced gene silencing can suppress the native gene.

Note added in proof

A third mechanism of generating functionally homozygous herbicide-resistant individuals has just appeared. It is similar to the approaches of gene conversion therapy being applied to mammalian systems. Single point ALS-resistant maize plants were engineered by converting the endogenous genes using chimeric RNA/DNA oligonucleotides (Zhu et al., 2000). This technology can be considered more as an in situ site directed mutagenesis more than transgenesis, as there are no exogenous DNA pieces remaining after the gene conversion is completed.

Resistance management – the need for stacked genes

Probably > 10 M ha of agricultural lands are infested with weeds that are resistant to one or more classes of diverse herbicide chemistry (Heap, 2000). Proactive resistance management is finally being considered; previously there was a smug assumption that industry would continue to develop many new chemicals. Now there is a realization that BD-HRC are almost all there will be new for a long while, and measures should be instituted to delay weeds from evolving resistance. One way to delay the evolution of herbicide resistance in weeds is to stack two genes for herbicide resistance and require that the farmers only use a mixture of herbicides. This is useful because it considerably lowers the mutation frequency for resistance in the weed. For example, if the frequency of resistance to one herbicide is 10^{-6} and the other 10^{-8}, the resistance to them stacked together is 10^{-14}, on condition that both herbicides are used. The use of stacking is very important where one wishes to preserve the ability of an excellent herbicide such as glyphosate, and one has a pernicious weed with a propensity to evolve resistance, e.g. *Lolium rigidum* in Australia.

There are two hazards that must be assessed with glyphosate and other BD-HR wheats: the risk of grass weeds such as *Lolium rigidum* evolving resistance to the herbicide, and the risk of introgression of the gene into related weeds. If glyphosate resistance is to be engineered into wheat, it should be stacked with a gene coding for resistance to a second unrelated graminicide. The herbicide mixtures should always be used when such wheat is cultivated. Glyphosate used alone will clearly further engender evolution of glyphosate resistance (Gressel, 1996; Powles et al., 1998; Pratley et al., 1999), and/or bring about a shift in weed spectra towards weeds that have never been controlled by glyphosate (Owen, 1997). The use of stacked BD-HR wheat and herbicide mixtures should delay the resistance of *Lolium* to glyphosate. The risk of introgression of stacked genes into wheat-related weeds will be discussed later.

When stacked resistance genes and herbicide mixtures are used, it is possible that there will be cases of enhanced weed control by the mixture. Still, some mixtures may be contraindicated vis a vis resistance management. Simply combining herbicides with different modes of action will not result in delaying resistance if the efficacy and temporal activity characteristics of the mixed herbicides do not match (Roush et al., 1990). Both mixing partners must effectively inhibit the weeds most sensitive to the vulnerable herbicide, because the greater the selection pressure on a weed species, the more likely it will evolve target site resistance (Gressel & Segel, 1982; Maxwell et al., 1990). Resistance could quickly evolve in a weed species that is naturally resistant to one of the herbicides in a mixture.

The components of the mixture need to have similar persistence or the mixing partner with a low mutation frequency must have longer persistence than the vulnerable one with a high mutation frequency of resistance. Otherwise, there will be a period when only the vulnerable partner is present, and it will select for resistance in the target weed as if there were no mixture at all. This would be the case when a persistent resistance-vulnerable ALS inhibitor is mixed with a short-lived phenoxy herbicide (Wrubel & Gressel, 1994). Unlike crops, which have been selected to germinate uniformly shortly after planting, seeds of many weed species display many flushes of germination during a cropping season (Bewley & Black, 1982). If a resistance-prone weed species has multiple flushes during the season, and the vulnerable herbicide has a longer period of activity than the mixing partner, then the vulnerable herbicide selects for individuals resistant only to it after the mixing partner has dissipated. A mixture that is not well matched for persistence can still be effective if all the weeds germinate over a short period of time, and both herbicides outlast the germination.

The ideal mixing partner should have three other properties (Wrubel & Gressel, 1994) in addition to equal persistence: (a) It should have a different target site of action from the vulnerable herbicide. (b) The

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mixing partner should not be degraded in the same manner as the vulnerable herbicide. For example, if the vulnerable herbicide is degraded in the crop by a glutathione transferase, the mixing partner should have no chemical site that can be attacked by the same enzyme. (c) Another useful attribute in a mixing partner would be to possess negative cross-resistance; i.e. where individuals resistant to the vulnerable herbicide are more susceptible to the mixing partner than the wild type. This would actually reduce the frequency of resistant alleles in the weed population. This strategy was first proposed for herbicides on the basis of laboratory data (Gressel & Segel, 1990b) and information on the existence of negative cross-resistance has been recently been published at the whole plant level (Gadamski et al., 2000).

Introgression of transgenes from crops to weeds

We have been warned that BD-HRC can lead to the evolution of 'superweeds' that will inherit the earth (Kling, 1996). The rapid commercial release of such crops has often been without broad-based scientific scrutiny with the most competent experts being involved, and as there are few published physiological, biochemical, ecological, genetic, and agronomic data to scrutinize. This leads to a certain degree of skepticism among scientists, which contributes to the public questioning the needs, utility, risks, and values associated with the use of BD-HRC. The severe pressures by detracting groups on policy makers makes it politically incorrect for pursuing public-sector research in this area, which prejudices the ability to perform experiments to obtain accurate information about the risks. These pressures also prevent generating crops needing resistance to herbicides where the agrochemical or seed industry perceives little profit.

Herbicide-resistant rice and wheat can be of great benefit only if used with care to prevent or mitigate gene transfer to related feral and wild rices and related weeds of wheat such as *Aegilops cylindrica*. Whereas the wild and feral rices are widely distributed, *A. cylindrica* has a relatively narrow distribution, although it can be quite pernicious. No solution in agriculture has been forever; farmers always had to deal with evolution. More sophistication will be needed than is presently being used with transgenic wheat and rice to mitigate introgression delaying such evolution.

Discussions of BD-HRC have rarely dealt with the risks from a weed biology perspective. The main risk stated by the detractors is claimed to be that of the BD-HRC becoming 'volunteer' weeds (in following crops), or their introgressing traits into a wild relative rendering it weedier; the 'super weeds' of the mass media. An attempt at such an assessment based on weed science was recently made using a defined set of uniform criteria in a decision tree format (Gressel & Rotteveel, 2000). Decision trees, by requiring discrete answers to sequential, stepped questions, lower the bias in arriving at conclusions vis a vis the risks deriving from a given hazard.

Conversely, there are wild species that are unlikely ever to become weeds unless they evolve a large number of weedy traits (Keeler et al., 1996). Unfortunately, too many risk studies do not differentiate between weedy relatives of crops and wild relatives (e.g. Sindel, 1997). Risk assessment must be performed on a local or regional basis, as the risks from the same BD-HRC will vary greatly from one agricultural ecosystem to another. A second assessment should be done (but has not been done in the past), about the effects of introgression from transgenic crops on weed flora of countries that import bulk unprocessed commodities such as wheat and oilseed rape (Gressel, 1997b).

The risks of introgression have been assessed on a case by case basis by regulatory authorities. The Canadians delineated criteria before even having to evaluate plants with novel traits irrespective of whether or not transgenic (Anonymous, 1994a), and then specifically evaluated oilseed rape in the context of these criteria (Anonymous 1994b). In a series of documents they further evaluated imidazolinone (Anonymous, 1995a), glyphosate (Anonymous, 1995b), and glufosinate (Anonymous, 1996) resistant oilseed rapes. The decision process was based on their perception of the risks to the regional agricultural ecosystems in western Canada, without considering other regions that may be importing the crops. Internationally the OECD (Organization for Economic Co-operation and Development) and UNIDO (United Nations Industrial Development Organization) are developing a series of 'consensus documents' on the biology of various crops (with regard also to related weeds) so that there is a common starting point to evaluate each cropping situation (Anonymous, 1997b). Most of the stated hazards of interspecific introgression from BD-HRC are based on unpredictive laboratory experiments, which prove that introgressions could occur. Thus they show that the hazard exists, but give little indication of risk; how quickly such transfers will occur in the field or

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how fit recipients will be to cope with competition in a multi-factorial situation. The time factor is not inconsequential; if resistance introgresses to produce resistant populations more slowly than natural mutational evolution of resistance, what is the significance of introgression?

Vertical, horizontal and 'diagonal' gene transfer

Two types of gene transfer are widely discussed: (1) vertical, within a species; or (2) horizontal, transfer among unrelated species, usually by asexual means. Biology is not as clear-cut; there can be some sexual transfer between plant species in the same genus and closely related genera. These are typically included in discussions of introgression as horizontal gene transfer. Horizontal gene transfer via plasmids is common in prokaryotic organisms. As extrapolations are often made from these rare cases of gene transfer among closely related species to 'prove' that all horizontal transfers are possible, I suggest terming these special interspecific cases in plants as 'diagonal' gene transfer, denoting the gray area where they exist.

Vertical and diagonal transfer possibilities are obvious to any biologist, but horizontal transfers, in eukaryotes, with their potentially disastrous implications to agriculture are not. The possibilities of true horizontal transfers are extrapolated from the intergeneric and inter-familial plasmid-mediated transfer of traits among microorganisms, which have allowed transfer of antibiotic resistance (analogous to herbicide resistance) among unrelated pathogens. The claim continues that because plasmids are often used as vectors in the genetic engineering of crops, inter familial transfers will become commonplace, or at least inevitable. This claim does not stand up to epidemiological experience with organisms such as *Agrobacterium tumefaciens* and *A. rhizogenes*. The plasmids used for laboratory gene transfers from these *Agrobacterium* spp. naturally infect a broad range of dicots, using the plasmid as part of the infection process.

If such inter-familial transfers were to occur via *Agrobacterium*, they would have been seen over the past 50 years with naturally occurring herbicide resistances. There are no known cases where such genes have transferred inter-familialy from any crop to weed via *Agrobacterium*, despite the great selective advantages that such weeds would have, and the ubiquity of *Agrobacterium* in the environment. The more than 10 million hectares of herbicide-resistant weeds that have appeared in the past 30 years can all be traced to evolution resulting from mutant selection, and not to plasmid-mediated horizontal gene transfer. Additionally, an extensive survey of all the Genebank database found few *Agrobacterium* DNA sequence pieces in any of the plant genes, which would have been expected in the millions of years of co-habitation. This matching task took hundreds of hours of computer time (Rubin & Levy, 1999). Horizontal gene transfer need not be discussed further; diagonal gene transfer is a hazard for which the risks must be estimated in particular cases.

Generalizing from hazards to risks

Because of genetic variability of crops and weeds, and the chemical variability in herbicides having different effects and modes of action, one cannot make easy generalizations about the risks of introgression of resistance. Each case needing a prediction of the risk of introgression must be evaluated on its merits, often after basic biological, genetic, and epidemiological studies. More importantly, other issues must be considered:

(1) What is the benefit to agriculture of introducing resistance into a certain crop, especially in management of evolved herbicide resistance in weeds?
(2) What are the possibilities of, and implications from, having herbicide resistance pass from the crop into a weedy or wild species?
(3) What are the possibilities of and implications from having the BD-HRC becoming a volunteer resistant weed in agricultural ecosystems, or an alien weed in ruderal or more pristine ecosystems?

The final decision on risk/benefit is ultimately a balance between science, economics, local benefits, local values, pressure groups, as well as local politics. The politicians often misuse or ignore science for clearly political decisions (Powell, 1997). Still, there is good reason that the criteria for risk assessment of BD-HRC should be uniform, using universal criteria and processes of examination.

Risks of introgression of transgenes to related weeds

In most of the world there can be virtually no risk of transgenes carrying herbicide resistance moving from crop to related weeds in such major crops as maize, soybeans, potatoes and cotton. Outside of the centers of origin there are no related weeds or even remotely interbreeding wild species that could become weeds. Clearly experimentation would be required in

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the centers of origin to assay the risk, but these centers represent a minuscule portion of the areas where these crops are grown.

Genes from wheat easily introgress into the genomes of some related wild species, wild species that are related to the progenitors of domestic wheat. There is much recent information of introgression of genes from hexaploid wheat into *Aegilops cylindrica*, a very problematic weed in the western plain states and the Pacific northwest of the USA (Zemetra et al., 1998; Seefeldt et al., 1998). This species shares a D genome with hexaploid wheat and homologous recombination occurs naturally under field conditions. It would theoretically be much harder to obtain transfer resistance from AABB tetraploid (durum) wheat to this species, as durum lacks the D genome. Thus, homeologous recombination (crossing over between related but dissimilar chromosomes) would have to occur when herbicide-resistant durum is used.

Genes for various traits have been transferred from many wild grasses to domestic wheat, especially from the genus *Aegilops*. Many *Aegilops* species are in fact considered to be *Triticum* species (Kimber & Feldman, 1987). The only other *Aegilops* spp. besides *A. cylindrica* known to have a wheat-homologous genome is *A. squarrosa* = *Triticum tauschii* (Kimber & Sears, 1987). Only homeologous recombination can occur between either hexaploid and durum wheats with the other less-related species. It takes much more than a single gene, even one for herbicide resistance, to turn a wild species into a weed (Baker, 1974, 1991). The risks of introgression of transgenes from wheat to these wild species are very low, even though breeders have moved them. Many intergeneric hybrids generated by breeders do not occur in the field; natural alleles have not passed from wheat to wild, related but mainly ruderal species and vice versa. The breeders have to resort to forced crosses, and then use techniques such as embryo rescue in tissue culture to save the hybrid embryos that would otherwise abort.

Most oilseed rapes cultivated are *Brassica napus*, a recent tetraploid derived from the CC genome of *B. oleracea* and the AA genome of *B. campestris* = *B. rapa* (U, 1935). Thus, the only weed where homologous recombination can occur is *B. campestris*. Still, homeologous recombination is known, especially in the laboratory, with many related species. Interspecifically this required hand pollination after emasculation of the weed, male sterility or self incompatibility in the weed, massive amounts of crop pollen, and/or embryo rescue of the rare progeny, that are mostly sterile or runts (Darmency, 1994a; Scheffler et al., 1995; Brown & Brown, 1996; Bing et al., 1996; Landbo et al., 1996; Lefol et al., 1996a, b; Mikkelsen et al., 1996). Initially, the general fear of BD-HRC precluded performing such experiments in the field especially in Europe where the public fears are greatest. This hysteria prevents obtaining field data that might substantiate, or more likely allay the fears of introgression. A large-scale field experiment in Australia found no introgression from oilseed rape to the related *Raphanus raphanistrum* (Rieger et al., 1999).

The significance of laboratory introgression studies to the field situation was evaluated by Karieva et al. (1996). The older epidemiological / apocryphal reports are actually more relevant to risk analysis than many of the artificial laboratory experiments. The older results could indicate that such transfers can occur in the field, as well as the time until predominance, and the competitive advantage (if any) of such introgressions. Few studies dare to comparatively estimate how long it will take to have resistance introgress and predominate in field weed populations vs. how long it would take resistance to evolve by natural selection, vs. the expected commercial lifetime of the herbicide.

It has already been shown that transgenic glufosinate resistance can be transferred genetically from rice to red rice (Sankula et al., 1998; Linscombe et al., 1998). How easily this will occur in the field is unclear, as rice is predominately self-pollinated, before flower opening. Less is clear about the other rice species. Rice is a species where there is far too little field information despite considerable information in the breeders and cytogeneticists laboratories. Cultivated rice *Oryza sativa* has an AA genome as does the red and many feral forms of weedy rice that are also *Oryza sativa* (Morishima, 1984; Aswidinnoor et al., 1995; Maesako et al., 1995; Brar & Khush, 1997; Aggarwal et al., 1997; Khush, 1997; Nakagahra et al., 1997). Genes readily move between the cultivated and feral forms despite rice being predominantly self-pollinating (Langevin et al., 1990; Aswidinnoor et al., 1995; Mariam et al., 1996; Brar & Khush, 1997; Majumder et al., 1997; Kaushal & Ravi 1998). There are many wild and weedy rice species, one of which, *O. rufipogon* has an AA genome, and is considered a major weed of rice (Holm et al., 1997). Another major weedy rice, *O. officinalis* has a CC genome. There are many other diploid and tetraploid wild (but not weedy) rices bearing genomes through the alphabet from AA to HHJJ. It is possible to transfer genes from many of these wild and weedy species to rice, which

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is how the breeders introduce new traits, despite the chromosomal incompatibilities (Brar & Khush, 1997; Khush, 1997). This often requires embryo rescue and/or intermediate crosses through bridge species. The significance of this to field problems is unclear. The taxonomic differentiation among these species is also rather unclear. In many places where weedy rices are even major problems it is not known whether the weeds are the con-specific red and feral forms or the other species (Cohen et al., 1999; Baltazar & Janiya, 1999). Indeed, recent studies have shown that the material in genetic resource depositories has been mis-classified. Much of this ambiguity will be clarified by modern molecular taxonomic techniques using DNA fingerprinting (Martin et al.,1997; Suh et al., 1997), so that better predictions can be made of risk in different locales.

Assaying introgression in the field

There are ways to ascertain the rapidity of gene movement without causing lasting damage to agriculture and/or the environment: simply insert a gene conferring resistance to a rarely used herbicide (Gressel & Rotteveel, 2000). There would be little consequence of the herbicide becoming unusable due to the resistance disseminating into the wild, as herbicides are not used on wild populations. Another tracing system could be used with acetolactate synthase (ALS) resistance. At present, if a weed becomes resistant to any ALS herbicide, it cannot be known whether it evolved resistance naturally by mutation, or was introgressed through cross pollination with a non-transgenic BD-HRC. The highly mutable ALS gene (ca. 10^{-6} natural resistance frequency in populations) quickly evolves naturally in weeds (Saari et al., 1994). Engineering the same ALS allele with either a two base change coding difference from the natural resistance allele, or with different introns, would allow easy differentiation between mutational events and introgression. This would indicate the rate of evolution due to introgression vs. the rate of evolution from natural mutation.

Triazine resistance has been found to be maternally inherited in many species, so one might assume it will never introgress from crops to weeds (Daniell et al., 1998; Daniell, 1999). Maternal inheritance is not absolute; 0.4% pollen transfer of triazine resistance was found with genetic markers in weeds (Darmency, 1994b) and 0.5% for tentoxin resistance in tobacco (Avni & Edelman, 1991). Maternal inheritance of chloroplast-encoded traits is typical, but there are many cases in higher species, including some crops where such traits are biparentally inherited (Tillney-Bassett & Abdel-Wahab, 1979; Corriveau & Coleman, 1988; Reboud & Zeyl, 1994). Corriveau and Coleman (1988) developed a rapid cytological assay to screen for biparental inheritence, which they did not find in the major agronomic crops, but did find in sweet potato, chick-pea, vetch, alfalfa, common beans, and geranium. In all, they found evidence for bi-parental inheritance in 14% of the 192 plant species assayed. Their evidence and the genetic evidence they cite would not have found the 0.4% paternal inheritance described by Darmency (1974b). Thus, it will be necessary to ascertain frequencies of paternal inheritance with each crop situation where maternal inheritance is predominant. There is a good chance that more pollen transmission will be found with herbicide resistance, and the estimates will be more accurate. Herbicide resistance is a better selectable marker than those previously available to the geneticists dealing with chloroplast inheritance, and because of the field size experiments, the numbers of plants tested will be much larger than had been possible. PCR amplification of chloroplast primers discriminate between *Brassica napus* and *B. rapa = campestris* in the ca. 0.6% natural hybrids that occur between the two species (Scott & Wilkinson, 1999). A low number of hybrids were examined (47) and all were indicative of maternal inheritance. It would have been far more interesting if they would have used chloroplast DNA encoded triazine resistance, using a semi-dominant nuclear marker to verify the hybrid nature of random field crosses of both species. The analysis would be far easier, and could be confirmed by PCR amplification.

Susceptible weeds growing amongst BD-HRC will not introgress the resistance genes if the herbicide is used; dead weeds don't have sex, but the genes could introgress into nearby unsprayed weeds. Seed set on emasculated plants from oilseed rape was measured 1.5 km from pollen source (Timmons et al., 1996), but can the progeny compete, or survive in feral populations without selector? Without emasculation of the recipient, resistant pollen fertilized 24% of conspecific plants in the immediate vicinity but < 0.027% just 10 m away. If BD-HRC seeds carry over as volunteers to other crops where related species are serious weeds, gene exchange might happen through this route and long distance dispersal becomes unnecessary.

Many weed scientists were surprised that the Canadian authorities allowed the field use of ALS inhibit-

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ors, glyphosate, glufosinate, (and soon) bromoxynil-engineered transgenic oilseed rapes. The surprise was due to the known introgression of herbicide-resistant genes into weeds, including the problematic *Brassica campestris = B. rapa* (e.g. Kerlan et al., 1996; Mikkelsen et al., 1996; Lefol et al., 1996a; Snow et al., 1999). *Brassica campestris* has been domesticated to become various crops in many places (Polish oilseed rape, turnip, Chinese cabbage, and pak choi). The more ancient con-specific wild form as well as the feral forms are pernicious weeds in other areas (Holm et al., 1997). While botanically identical, they have very different morphotypes, with very different phenologies, biologies, and competitiveness. Most of the *B. campestris* crop types are easily controlled in fields, and as easily controlled volunteer weeds, they have never left agricultural or ruderal areas. Their botanically, but not phenotypically identical weedy twin can be very problematic in agro-ecosystems, and was a predominant weed in grains before the advent of selective herbicides (Holm et al., 1997). It is hard to predict what will become of the feral populations of Polish oilseed rape, which still remains ruderal. They could evolve to become more weedy.

Deleterious weed gene introgression from feral populations into both commercial oilseed rapes is common, lowering yield and oil quality (e.g. McMullen et al., 1994). A major use of herbicide-resistant oilseed rapes is to facilitate control of its wild relatives. The Canadian authorities subjected non-transgenic ALS-resistant oilseed rape derived by mutagenesis to full regulatory scrutiny before release to the market. Their decisions (Anonymous, 1995a, b; 1996a, b) allow unrestricted field cultivation, while noting the likelihood of introgression into *B. campestris*, and stating that the worst case would be the loss of the particular herbicide to control such weeds (Anonymous, 1995a). The decision, partly based on release studies (Crawley et al., 1993) stated that introgression would not increase weediness of the crop or related weeds outside of agriculture. *B. campestris* is the weediest of species related to oilseed rape (Holm et al., 1997), and is the one species that demonstrated field transfer of genes (Mikkelsen et al., 1996), but not quite as readily as initially presumed (Lando et al.,1996). No formal or informal monitoring system was instituted. This is unfortunate as much has been written on how comparatively easy it is to eradicate small pest populations, and how impossible it is after they have reached a critical size (Moody & Mack, 1988; Thill & Mallory-Smith, 1997), yet few learn these lessons.

Instead, a recent survey has shown widespread introgression of herbicide resistance traits among the various volunteer herbicide-resistant oilseed rape populations in western Canada (Hall et al., 2000). Many volunteer populations already contain all three released herbicide resistances, to ALS inhibiting herbicides, glufosinate and glyphosate. The regulatory system cannot preclude re-introducing triazine-resistant varieties, and possibly could not prevent the use of 2,4-D resistant varieties if such were generated using known transgenes (Streber & Willmitzer, 1989), because each case is considered on its own merits. There soon could be 'volunteer' weed populations of oilseed rape in subsequent crops in rotational cycles that cannot be controlled by any herbicides available in the farmers' arsenal. There is clear reason to consider requiring the use of certified seed for all plantings along with a requirement that the certified seed contains a single herbicide resistant gene, unless there is premeditated reason for stacking and a requirement for the use of herbicide mixtures.

The question of how quickly resistance genes will move from *B. napus* to *B. campestris* may be moot, as Polish oilseed rape (*B. campestris*) with various herbicide resistance genes has also been released in western Canada. The crop as a volunteer weed could become a problem, especially if it also becomes multiply resistant via cross breeding to all four herbicides introduced in various varieties.

Preventing and mitigating introgression

There are various failsafe mechanisms that can be used to prevent or mitigate the risk of introgression, when and if it does occur (Gressel, 1999a). These vary from management practices (weed free zones around transgenic crops) to techniques that involve breeding, or more biotechnology. Those using a transgenic approach are discussed below, but other approaches such as using apomyxis are also conceivable (Gressel & Rotteveel, 2000).

Gene placement failsafes

The particular placement of a transgene in crop genomes can affect its movement to other varieties and species.

Chromosomal

Wheat and oilseed rape are composed of multiple genomes derived from different wild sources (U, 1935;

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Kimber & Sears, 1987). In any given locale, it is possible that only one of the genomes of the crop is identical to that of a related weed allowing easy gene transfer. As the D genome of wheat is compatible with the D genome of *Aegilops cylindrica* and the B genome of oilseed rape to many brassica weeds, transgenes easily introgress from them to wild species. One can perform the cytogenetic localization to assure that the transgene is on the incompatible A or B genomes of wheat or the C genome of oilseed rape, and then ascertain if there is no homoeologous introgression, i.e. crossing over between the non-homologous chromosomes. There is evidence though for a considerable extent of homeologous introgression between oilseed rape and *B. campestris* (Mikkelsen et al., 1996), so there is no utility in this type of failsafe in oilseed rape, but there is little information for assessing homeologous wheat introgression into *Aegilops cylindrica.*

Hybrids

A simple failsafe can be found with hybrid crops. If a dominant transgene for herbicide resistance is placed in the male sterile line in close linkage with the male sterility gene, there will be no possibility of introgression in crop-production areas. Care will only have to be taken in the seed production areas when the male sterile line is restored. Such areas must be kept free of related weeds, a typical precaution in seed production generally practiced before the advent of transgenics.

Plastome or chondriome

If the transgene for herbicide resistance is placed on the mitochondrial or plastid genomes, as has been done in tobacco (Daniell et al., 1998; Daniell, 1999; Kota et al., 1999; Khan & Maliga, 1999) there should be limited possibility of gene flow, due to the maternal inheritance of these genomes. Species such as tobacco that are often claimed to have no paternal inheritance often have about 0.1–0.5% pollen transfer of traits (Avni & Edelman, 1991; Darmency 1994b). The risk of transgenes being established in related populations is far greater with herbicide resistant traits where the herbicide exerts selection pressure than traits such as production of medicinals, with other traits in between. Large scale experiments should be performed with such crops to assure that the level of paternal transfer of traits is sufficiently low to justify using this strategy, and the risk analysis must consider the transformed traits.

Transient transgenics

It would be conceivable to insert certain metabolic traits (e.g. catabolic herbicide resistance) on RNA viruses or in endomycorrhizae that are expressed in the plant but are not carried through meiosis into reproductive cells. One would have to transfect the crop every generation, but the transgenes could not spread sexually.

Transgenetic mitigation (TM)

Genetic engineering can be used to mitigate any positive survival traits transgenes may confer. If the herbicide resistance gene engineered into the crop is flanked on either side by a TM gene in a tandem construct, the overall effect would be deleterious to weeds introgressing the construct from a crop (Gressel, 1999a). This is based on three premises:

(a) Tandem constructs of genes act genetically as tightly-linked genes and their segregation from each other is exceedingly rare;
(b) There are traits that are either neutral or positive for a crop that would be deleterious to a typical or volunteer weed, or to a wild species; and
(c) As weeds are strongly competitive amongst themselves, and have large seed outputs, individuals bearing even mildly deleterious traits are quickly eliminated from populations. Even if one of the TM alleles mutates, is deleted, or crosses over, the other flanking TM gene will remain, providing mitigation.

TM traits that could be used are best visualized when observing the differences between crops and weeds. This is best illustrated with two cases: (a) wheat and weedy relatives (b) oilseed rape (*Brassica napus)* and feral and weedy Polish rape/wild radish *B. campestris* = *B. rapa*, as summarized below.

Traits for transgenetic mitigation

Seed dormancy: Weed seeds typically have secondary dormancy, with seeds from one harvest germinating throughout the following season and over a number of years. This evolutionary trait is considered to be a risk-spreading strategy that maximizes fitness while reducing losses due to sib competition (Lundberg et al., 1996; Hyatt & Evans, 1998). Staggered secondary dormancy prevents all the weeds from being controlled by a single agronomic procedure. However, crops have lost secondary dormancy as a result of domestication.

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Genetically abolishing secondary dormancy would be neutral to both crops, but deleterious to their related weeds. Tillage, crop rotation, and preplant use of herbicides, all standard practices would control the uniformly-germinating TM weed seeds lacking secondary dormancy in rotational crops.

Ripening and shattering: Weeds disperse their seed over a period of time and much of the ripe seed 'shatters' to the ground, ensuring continuity. A proportion of the weed seed is harvested with crop seed, contaminating the crop seed and facilitating weed dispersal to wherever the crop seed will be grown. Weeds have evolved morphological and phenological 'mimicries' to the crop seed (Barrett, 1983; Gould, 1991), necessitating continual evolution and refinement of techniques to remove the contaminating weed seed. Recently domesticated crops such as oilseed rape still suffer from shattering (Price et al., 1996; Prakash, 1988; Simon, 1994). In addition to the loss of yield, the shattering of crop seed results in their becoming a volunteer weed, especially in oilseed rape (Lutman, 1993).

Uniform ripening and anti-shattering genes would be detrimental to weeds, but neutral for rice that ripens uniformly and does not shatter and positive for oilseed rape, which still has a shattering problem.

Dwarfing: Crops have been selected for height, to outgrow weeds. Weed evolution kept apace, selecting for taller weeds. The advent of selective herbicides to kill weeds allowed for genetic dwarfing of these crops, with more seed harvest, and less straw. Various new systems of genetically engineered height reduction are being introduced. These include genes relating to hormone production, (Azpiroz et al., 1998; Schaller et al., 1998; Peng et al., 1999) as well as those dealing with shade avoidance. Shade avoidance is advantageous when competing with other species, but not in a weed-free crop stand where only siblings are competing. The overexpression of specific phytochrome genes prevents recognition of shading and thus the plant remains short (Robson et al., 1996). This is advantageous for a crop and could also be used where the present dwarfing genes prevent obtaining the highest yields. Dwarfing would be disadvantageous for a weed that must compete with the crops. It would be shaded over by the crop.

Balancing primary and TM traits

If the primary transgenic trait confers an advantage to a weed, how much will TM traits actually mitigate that advantage? Weeds are not only highly competitive with crops, they are competitive with weeds of other species as well as within their own species. Weeds typically produce thousands of seeds in steady-state conditions, to replace a single plant, suggesting extreme competition to be the replacement; the selection for the highest competitive fitness is intense.

Other transgenes can be considered for mitigating the risks of integration with other crops. These include: (a) eliminating seed coat characters that allow weed seeds to pass through animal digestive tracks intact and then be dispersed; (b) genes promoting partitioning to roots would be advantageous to cultivated root crops such as beets but detrimental to related crops such as annual wild beets; (c) genes that prevent premature flowering (bolting), i.e. promote biennial growth, would be excellent for carrots, celery, cabbage, lettuce, beets, and related crops, but would be highly deleterious to related weeds; (d) anti-flowering genes would prevent introgression of genes from potatoes into its wild Andean relatives, and prevent volunteer potatoes arising from true seed.

After a weed introgresses a transgene and then stabilizes (eliminates cytogenetic incompatibilities), the trait will quickly spread through a population, even if it has a marginally positive fitness advantage (Thill & Mallory-Smith, 1997). Conversely, one can balance the disadvantage of TM traits against the advantage of the primary trait. This must be done in both in the presence and absence of the herbicide, as herbicide resistance only provides an advantage when the herbicide is used. Indeed, when the herbicide is not present, the transgene resistance trait can be disadvantageous as demonstrated with an ALS resistance transgene (Bergelson et al., 1996).

Each TM trait should work in a balance with the primary trait, and it might be necessary to have more than one TM trait in a construct to obtain balance. The risks of introgression can be further decreased by combining TM traits with a cytogenetic failsafe, where these are available.

Even if one or two TM genes were to confer some unforseen and unforseeable advantage in the future, this would be akin to evolving resistance to a herbicide. The first case would be reviewed, and a decision could be made whether the situation warrants removing the TM transgenic crop from market to prevent the occurrence of further cases. The only aspect that is predictable is the segregation of tightly linked genes, and that is why flanking the primary gene on both sides could be used. The segregation of a gene would be vis-

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able, allowing any such material to be removed from the breeding population.

TM genes are available to mitigate movement of resistance

Some possible traits for TM constructs just exist as named genes that are inherited, others are also mapped to positions on various chromosomes, and a few are actually characterized as sequenced genes. Thus, not all TM traits have genes that are immediately available for insertion in tandem constructs. Still, there can be many different ways for a plant to confer a TM trait, and thus, more than one gene might be available.

Secondary dormancy. Unfortunately, *Arabidopsis,* the typical source for genes, has already been sufficiently domesticated so that it is unlike cruciferous weeds; the laboratory strains no longer have strong secondary dormancy (Van der Schaar et al., 1997). A mutant that is insensitive to abscisic acid and lacks secondary dormancy was found in a wild, undomesticated *Arabidopsis* strain (Steber et al., 1998).

Shattering. Physiologically, one way to avoid premature seed shattering is to have uniform ripening. Early maturing seeds of oilseed rape on indeterminate, continuously flowering varieties typically shatter. Determinacy, with its single uniform flush of flowering is one method to prevent shattering, but this often shortens the season, reducing yield. The hormonology of the abscission zone controls whether shattering will occur and it is possible that if cytokinins are overproduced, then shattering will be delayed. As with secondary dormancy, no sequenced genes are yet at hand, except for cytokinin overproduction.

Dwarfing. Many of the genes used for breeding dwarfism seem to have an unknown function. Still many genes are known that control height.

Gibberellins. Preventing the biosyntheses of gibberellins reduces height (Webb et al., 1998), which is the basis of many chemical dwarfing agents used commercially on wheat. The three enzymes and genes controlling various steps in gibberellin biosyntheses are known and cloned (Hedden & Kamiya, 1997; Yamaguchi et al., 1998; Helliwell et al., 1998; Lange, 1998). *Arabidopsis* mutations bearing mutations in any of them are dwarfed, and the dwarfing is reversible by gibberellin treatment. Overexpression of a gene coding for *ent*-kaurene synthase, causing cosuppression mimicked the mutant phenotype. Additionally, a GA receptor gene has recently been isolated that confers gibberellin insensitivity when transformed into grains (*GAI*), and thereby induces dwarfing (Peng et al., 1999).

Some processes such as flower stalk bolting are controlled by specific gibberellins; in radish GA_1 and GA_4 are responsible (Nishijima et al., 1998). It may be necessary to characterize the genes coding for the monooxygenases and dioxygenases that are responsible for these later steps (Hedden & Kamiya, 1997). Some of these genes have been isolated as well (Kusaba et al., 1998).

Brassinosteroids. This group of hormones also causes elongation of stems in many plant species, and their absence results in dwarf plants. A 22 d-hydroxylase cytochrome P-450 has recently been isolated that controls a series of these steps in brassinosteroid biosynthesis (Choe et al., 1998) and plants lacking the enzyme are dwarfed (Azpiroz et al., 1998). Plants are also dwarfed when they produce normal levels of these growth regulators but are mutated in the *bri1* gene coding for the receptor (Noguchi et al., 1999). Additionally, suppressive overexpression of a sterol C24-methyl transferase in the pathway also causes dwarfing (Schaller et al., 1998).

Shade avoidance. Various forms of the pigment phytochrome interact to detect whether a plant is being shaded (Smith & Whitlam 1997; Torii et al., 1998; Devlin et al., 1998). The engineering of suppressive overexpression constructs of one of these phytochromes led to plants that did not elongate in response to shading (Robson et al., 1996).

Modifying crops and weeds to manage resistance without herbicides

There is more to biotechnology than developing herbicide-resistant crops as tools for managing herbicide-resistant weeds. Engineering the crop directly to be resistant to weeds without a herbicide is a concept that is mainly a glint in the eyes of researchers. One exception is the case of parasitic weeds, where genes have actually been found that confer resistance, both in maize and in its wild relatives, and are being engineered into maize (Grimanelli, 2000). Various researchers have proposed engineering allelochemical production into crops. One might question the metabolic cost of such production against final crop yield, and whether it will be economic, questions that can only be answered in the field. As a larger arsenal is needed to manage herbicide-resistant weeds, it is also worth considering engineering weeds to lesser

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virulence, or engineering biocontrol agents to greater virulence, as outlined below.

Transgenics can allow weed control with less herbicide

There is no need to assume that the only place transgenic research will assist agriculture in weed control is via herbicide-resistant crops. Other envisaged uses, extending towards science fiction, are described below. The reader is hopefully stimulated to envisaging other uses and acting upon them.

The systemic movement of amino acid biosynthesis inhibiting and related herbicides facilitates translocation to metabolic sinks. Unfortunately for the farmers, parasitic weeds are such metabolic sinks, sucking the plants dry. Small amounts of herbicide move with photosynthate to the parasitic weeds, facilitating their death. In an effort to render parasitic weed control affordable in the lesser-developed areas of the world, seed treatments were envisaged. Thus herbicides such as glyphosate could be primed into seeds to redistribute through the developing roots (Gressel & Joel, 1997) or various ALS inhibitor herbicides could be dressed to seeds, as primes, drenches or in polymer or dust coatings (Abayo et al., 1998; Berner et al., 1998; Kanampiu et al., 2000). Whereas less than 10% of the normal amount of herbicides used per hectare is used, and there is no need for spray equipment, the concentration of herbicide surrounding the seed is very large. Normally, metabolically-resistant crops could not withstand this high, localized herbicide level, it requires high levels of resistance afforded by modified target sites.

There may be spin-off from this research to the developed countries. Pre-emergence herbicides applied to soil surfaces in spring often become major environmental problems when the heavy rains carry them away as run-off contaminants. The major need for weed control at that time is in the immediate vicinity of the crop seed. Weeds germinating further away can be controlled at a later time. Thus, herbicide applied to crop seed facilitates 'cost-free soil incorporation', effecting a weed free clear zone near the crop.

Indirectly controlling weeds by facilitating crop mineral nutrition

In the last 50 years fertilizer use has greatly increased, increasing crop yields, selecting for weed species that perform better, and eliminating species that did not respond (Haas & Streibig, 1982), while causing environmental problems due to leachate. Unfortunately most plant breeding trials are performed under supra-optimal nutrition. A crop variety with greater nutrient efficiency and slightly lower yield may be profitable when fertilizer costs are calculated if such a new variety can be registered with present guidelines (because of lower crop yield, but with possibly higher economic yield). As environment factors become factored into the cost-benefit system, then nutrient utilization efficiency of crops may increase, allowing the crop to compete with less fertilized weeds. Concepts for using molecular techniques to enhance nutrient efficiency are advancing (Hirsch & Sussman, 1999), and weed control in such crops may be simpler.

Allelochemical weed control by crops

Various plant species are known to suppress other species by production of allelochemicals, chemicals not toxic to the originating plant but toxic to other vegetation. Transgenic allelochemical production is already being used to ward off pathogens; e.g. by transgenic production of stilbenes (resveratrol) by transforming with the gene coding for stilbene synthase (Fettig & Hess, 1999). This is a rare case of a secondary metabolite being produced in a single step that is one gene removed from a primary metabolic pathway. Additionally, it is always easier to chemically suppress an insect or pathogen than another plant species, where the relatedness to the crop is closer.

Most secondary metabolites that can be used as allelochemicals are very complex and a multi-gene system might have to be transformed into the crop to produce allelochemicals. Additionally, allelochemicals are typically short-lived in the environment; a crop would have to continuously synthesize them. This might be at considerable metabolic cost that would lower crop yield. Crops, like other plant species must 'choose' a survival strategy 'to grow or to defend' (Herms & Mattson, 1992). Many of the allelochemicals 'naturally' utilized by plants have considerable mammalian toxicity or carcinogenicity. It would be hypocritical to advocate using such compounds just because they are natural. Thus, one would hope that the release of plants producing them would have the same requirements by toxicity testing as the use of herbicides; daunting and costly requirements, but morally and scientifically justifiable. All the above is hypothetical. It would be wise to wait until more is known about the pathways of particular allelochem-

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icals. When the multi-genes become available, then produce transgenics to truly establish whether they have the utility and the safety needed.

Enhancing morphological competition with weeds

Based on theoretical considerations it was found in the field that mixing a group of barley varieties heavily reduced the influence of mildew (Wolfe et al., 1992). This was highly effective in the field until requirements for marketing 'pure' varieties were instituted. Perhaps crops can compete with weeds better if genes were separately introduced into strains of a variety that would change morphotypes and root distribution. The variety would be isogenic except for those traits and might even have higher yields, despite the plants having different appearances. Varietal registration authorities will have to modify their views on varietal uniformity before such concepts can be commercialized.

Forcing weeds to revert to being innocuous wild species

A solution, once thought to be in the realm of science fiction, is to transgenically debilitate the weeds that exist in farmers fields (Gressel, 1999b,c; Gressel & Levy, 2000). If such a solution is successful, it will integrate with and facilitate other successful control mechanisms, leading to more durable control. Modifying pests to facilitate their control is not novel. The release of irradiated male insects to impregnate females with sterile sperm has been used successfully in some venues. A transgenic approach, using a wide variety of possible genes that will be deleterious when turned on was proposed. The genes could mimic herbicide action, inhibit plant growth, render super susceptibility to herbicides, or modulate hormone levels.

The TAC-TIC approach

Pfeifer and Grigliatti (1996, 1997) proposed a means for controlling pests called the TAC-TIC model: 'Transposons with Armed Cassettes for Targeted Insect Control'. They proposed to transform insects with a gene, which if activated, would debilitate the insect. They proposed to use a chemically-induced promoter to activate genes that would prevent feeding, mating, or otherwise kill the insect, i.e. chemically-assisted-suicide genes as *kev* (Kevorkian) genes. They postulated that not many transgenic pests would be needed if the transgenes are transmitted in multicopy transposons, which will quickly disperse in the indigenous population through mating. They suggested that the farmers could use pesticides during the period of transposon dissemination. As no method of control kills all pests, the deleterious transposon (DT) containing and lacking individuals will be similarly affected. The transposons would disseminate among the remaining pests, as they would in a population without pesticide. After transposon transmission is complete, the use of a chemical that turns on the chemically-induced promoter replaces the pesticide.

Such an approach could be modified and work with pernicious weeds such as *Lolium rigidum, Echinochloa crus-galli* and *Striga hermonthica*, which are solely or predominantly outcrossing. Indeed, if the proper *kev* genes and promoters can be found, and the transposons available; these species could be easily engineered. Most importantly, biosafety considerations must be dealt with.

The *kev* genes can be introduced into a transposon cassette and transformed into the weeds to generate partially debilitated plants after chemical induction, or possibly before induction. Transposon proliferation should produce high copy numbers of the *kev* genes in the progeny of these sown parents. The debilitating genes will be dominant and high copy number. Therefore, most if not all the progeny will rapidly contain them. The *kev* containing weeds could then be abundantly sown (as in *Lolium* pasture) to increase the frequency of *kev*-containing plants in the field.

The Ac/Ds transposon family, originally found in maize (McClintock, 1951) has been shown to be active in all the heterologous plant systems (approx. 15 different species) where it has been introduced (Kunze, 1996). Ac is preferentially transposed during DNA replication, and as a result its copy number can increase while it transposes (Greenblatt, 1984).

Potential kev *genes*

In the initial TAC-TIC concept, no specific genes were suggested for use. Perhaps the unavailability of appropriate genes (and promoters) stifled further development of the concept. A plethora of potential genes are available 'off the shelf' for use in weeds.

One would expect *kev* constructs to be perfectly useful on the *Lolium* biotypes that evolved herbicide resistance by enhancing metabolic pathways that degrade a multitude of herbicides. One could envisage inhibiting a joint promoter region for a plethora of cytochrome P-450 to suppress some multiple herbi-

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cide resistances, allowing again weed control by the previously used herbicides. *Kev* constructs might not be as effective on weed populations that have evolved herbicide resistance by overproducing the same target gene product as is targeted. It has been claimed that a glyphosate-resistant *Lolium* strain produces double the normal level of EPSP synthase (Gruys et al., 1999), as do resistant *Lotus corniculatus* strains (Boerboom et al., 1990). A *kev* gene inhibiting EPSP-synthase might be less effective on such biotypes.

Chemisterilant kev *genes*

Chemically-induced genes that will cause pollen sterility a generation hence have been proposed for protecting crop varieties (Crouch, 1998; Masood, 1998; Oliver et al., 1998), the so called 'terminator genes' of the popular press. Similar constructs could be considered for use as *kev* genes, when disseminated by transposons. This could easily be used in *Lolium* planted in pastures, with the terminator gene turned on prior to planting wheat (but not in areas used to propagate seed). This would always require sowing pastures.

Debilitating kev genes. Weeds have evolved to be highly competitive, both with the crop as well as with other weeds and siblings. A typical weed plant produces thousands of pollen grains to fertilize one ovule, and hundreds of seeds to replace one parent. Thus, the competition within the species is quite fierce, both to fertilize and during the 'self thinning' period when seedlings establish. Some of the genes proposed for use in TM constructs could be used as DT genes in weeds as they would lower fitness.

As, *Lolium* has evolved more herbicide resistances than other weeds, we will discuss mainly how *Lolium* can be controlled with this approach. Many of the same genes could be used to manage herbicide-resistant *Echinochloa* as well. *Kev* genes for *Striga hermonthica* have been discussed elsewhere (Gressel & Levy, 2000; Gressel 1999c). Non-chemical strategies would be appropriate for the three interbreeding *Lolium* spp; (Gressel, 1999b); *L. rigidum, L. perenne* and *L. multiflorum*, which are very important pasture grasses in rotation with, or near grain fields. There would be nothing wrong with *Lolium* in pasture phase containing potentially self-lethal *kev* genes, as long as the chemical inducer is not leaky i.e. cannot get turned on in the pasture phase. An additional or alternative approach can be considered with *Lolium*. One can use *kev* genes that are neutral or even positive during the pasture phase but decidedly deleterious during the cropping phase. Thus, *kev* genes that cause dwarfing by internode shortening or non-recognition of shade would not matter in pastures. Genes that severely affect vertical structural stability such as those strongly suppressing lignin formation would also increase palatability as well as vastly increase the amount polymeric carbohydrates that can be digested by ruminant animals. Genes suppressing auxin biosynthesis or stimulating cytokinin biosynthesis would suppress the dominance of the shoot apex, stimulating branching (tillering), which is good in pasture but is deleterious in competition with crops. Suppression of long term (secondary) dormancy could be added in addition to preclude remaining in the seed bank for more than one season, ensuring that seeds will not remain overlong in the soil, perhaps even requiring annual storage and sowing. All these genes will be beneficial to the pasture species but will render it less competitive with a wheat crop, even a very dwarf wheat crop.

Lolium has an additional advantage. Whereas few weeds have been transformed, two species of *Lolium* has been shown to be amenable to straightforward transformation techniques (Spangenberg et al., 1995; Ye et al., 1997). Clearly these procedures were elucidated because of the importance of *Lolium* as a pasture species.

Chemically induced promoters

The TAC-TIC concept for insects requires the use of chemically-induced promoters, although no specific examples were listed in the initial papers. A wide variety of promoters are available for chemically inducing the expression of genes in plants (Gatz, 1997; Gatz & Lenk, 1998). Some inducers are antibiotics or expensive compounds deemed inappropriate for agronomic use, but copper salts and ethanol are among the inexpensive, simple inducers for known promoters.

Biosafety of using deleterious transposons

Weeds in their present evolutionary state are not much more competent than crops to exist in the wild. As with crops, when a weed becomes feral, it can at best usually exist only in human-disturbed (ruderal) ecosystems. With DTs, evolution is reversed. Most weeds would be forced to revert from their highly evolved specialized status in agroecosystems to their generalist status as innocuous wild plants. DT constructs could force the evolution of *Lolium* back to being a superior pasture grass, but not a weed. When *kev* genes are used under a chemical promoter, there

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should be little danger accruing from the DT transposons entering wild populations of the weed species or its introgressing relatives. The DT transposon should have little negative value if the chemical inducer of the DT gene is not found in the wild.

The above are general theoretical considerations, but it is clearly necessary to perform a risk analysis based on the particular weed, its place in agro- and natural ecosystems, and whether it has readily introgressing relatives. The genes used could only confer a negative value on the weed, and it might disappear from fields. Clearly until one knows how such genes will act, one should try such technologies with introduced weeds, far from their centers of origin. One must consider the potential risk from different *kev* genes under the control of different promoters. One could consider constructing decision trees, similar for those constructed to estimate the risk of transgenes introgression from crop to weed to deal with such issues (Gressel & Rotteveel, 2000). Clearly, despite paper analyses, experiments should be designed to safely evaluate the risk of environmental hazard (if any) from DT weeds.

Engineering biocontrol agents to greater virulence for controlling resistant weeds

There are many cases where there is a need to control a single naturally or evolved herbicide-resistant weed species, while all other species are adequately controlled by cost-effective means. Either especially high rates of herbicide are needed to control such resistant weeds or an expensive herbicide is needed. If the single problem weed species could be controlled by non-chemical means, then lower, more economical and less potentially polluting rates of the herbicide could be used to deal with the easier to control weeds. In such a case biological control is appropriate because it can potentially allow selectivities unavailable, cost-effectively with presently available chemicals. Pathogens are typically specific to single host species or to a few related hosts, which should allow the necessary selectivity between crop and weed host. There are many reports of mycoherbicidal agents, as well as excellent books on the biocontrol of weeds (cf. Te Beest, 1991), but commercial successes are limited for reasons discussed by Auld and Morin (1995) and Weston (1999). The typical cases use indigenous agents that just make plants sick, but do not kill them. A truly 'inundative' approach is required, using a heavy inoculation (typically $> 10^4$ spores cm^{-2}) to adequately control weeds. From an evolutionary standpoint the inundation is understandable. If an organism was hyperpathogenic, both it and the weed host might have become extinct or at least the weed would be diseased enough to render it to be just another wild but not weedy species. Thus, ways must be found to circumvent the need for heavy inoculation, or else biocontrol cannot be economically sound. Most industrial efforts have sought to produce and formulate the spores used as inocula less-expensively, instead of finding ways to use less, or to render them more efficient.

There are two types of reasons for the need of inundations of fungal material: (a) There can be inherent problems in getting the organism to the right place and keeping it alive until an adequate infection is established. There are problems in getting soil and stem pathogens to their inaccessible targets. Leaf and stem pathogens often need 6–18 h dew periods to germinate and become established. Such optimal conditions do not often exist in nature, which is why many crop disease pandemics are sporadic and weather related; (b) Despite a pathogen being 'compatible' with the weed, it does have to overcome the weed's inherent defense mechanisms. This is a 'quantitative' battle; i.e. a function of the level of inoculum used.

The attacked weeds initiate various defenses to suppress the spread of invading pathogens including: (i) PR (pathogenicity-related) proteins (chitinases and glucanases as well as other stress-related proteins). The chitinases and glucanases degrade fungal cell walls, preventing penetration and growth in the tissue; (ii) phytoalexins, non-protein, secondary metabolites of various chemistries that poison the fungus; (iii) physical barriers composed of polymers such as suberin, lignin, callose, or their mixtures. Often papillae of callose rapidly form, blocking the advancing fungal hyphae. Such papillae can contain small amounts of lignin. Traces of lignification can severely suppress degradation of carbohydrate polymers such as callose and cellulose by fungal enzymes (Gressel et al., 1983). Different species initiate different defenses, and the levels of induced and pre-existing defenses vary during weed growth and development. The outcome of this is that different quantities of inocula are needed at different stages of growth, a major complication to using biological control agents. Similar age dependencies often exist with herbicides.

Synergizing biocontrol agents to control weeds

Keeping an organism alive and then getting the biocontrol agent to the right place and keeping it there

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is the task of formulants used to obviate the need for extended dew periods (Boyette et al., 1991). Typically spores are used and few successful attempts have been made to produce and store fungal mycelia alone. Late log phase liquid fermenter cultured isolated mycelia of two *Fusarium* spp. specific to the parasitic *Orobanche* spp. were formulated in 'Stabileze' (starch, sucrose, maize oil, and silica) and air-dried. 'Stabileze' formulations exhibited little loss of mycelial viability and retained pathogenicity for over a year (Amsellem et al., 1999). Chopped mycelia from liquid culture yielded 40 times more colony forming units than spores of one organism. Efficient formulation of mycelia should significantly change the biosafety and economics of biocontrol, with spores no longer needed, and liquid fermentation the preferred method of mass-producing inoculum. The outgrowth of rehydrated mycelia is faster than spore germination, with mycelia attaching to and penetrating the host more quickly than germinating spores.

Chemicals, especially herbicides, have been used, some seemingly 'off the shelf', to ascertain if they will synergize mycoherbicides and bacterial agents used for biocontrol of weeds (Christy et al., 1992; Gressel et al., 1997). This requires an understanding of the defenses being used by the pathogens. This approach can work successfully, at least at the laboratory level where it was found that inhibitors of the phenyl-propanoid pathway could inhibit phytoalexin production in a legume weed (Sharon et al., 1992), and chemicals that bind calcium prevented the action of callose synthase in *Abutilon theophrasti* (Watson et al., 2000). In both cases the infection by specific pathogens was enhanced by the treatment with the chemical synergists.

Organisms can be potentially modified to increase pathogenicity by genetic transformations with genes for virulence from other species, by increasing the endogenous expression of genes, or by transfer from other organisms by protoplast fusion (cf. Harman & Stasz, 1991; Kistler, 1991). Increasing virulence, especially by gene transfer, requires extreme care due to environmental impact, i.e. the possibility of increasing the host range to include other crops. Groups are considering engineering 'fail safe devices' into their pathogens. Conversely, highly virulent, not too specific organisms can be 'disarmed' to allow them to be used where there is crop selectivity. For example, a widespread broad-spectrum pathogen *Sclerotinia* was mutated to auxotrophy and to be sclerotia minus, i.e. it could only reproduce vegetatively in culture, with the missing nutrients added. It could still infect weeds having the missing nutrients, but 'commits suicide' when the weed dies (Sands & Miller, 1993). The use of formulated mycelia instead of spores (Amsellem et al., 1999) would allow the effective use of asporogenic deletion mutants, which would limit dissemination of biocontrol organisms, as well as prevent them from overwintering or living through other periods of stress. This failsafe against spread should allow engineering toxin production into the biocontrol agent, or engineer genes that disrupt the weed growth or metabolism to enhance virulence. The specificity of the biocontrol organism would prevent attack of crops, and the use of formulated asporogenic mutants would prevent spread.

Thus, with adequate formulation and genetic modification, it should be possible to synergize biocontrol agents to lower inoculum levels and enhance virulence, allowing specific control of weeds that have evolved herbicide resistance (as well as other weeds). There are theoretically 2–5 orders of magnitude for improvement with most such agents. One order of magnitude renders most biocontrol agents cost-effective.

Concluding remarks

Whereas molecular biology is fixated at present on the immediately commercializable aspects of chemical weed control (functional genomics and BD-HRC), one can envisage many other ways that genetic engineering can be used to partially replace or augment herbicides in controlling weeds. Thus, it is deplorable that because of an anti-chemical bias among some of the authors of the World Bank report, weeds were not among the constraints to agriculture that could be overcome by biotechnology (Kendall et al., 1997). By not listing weeds, many biotechnologists do not realize the problems facing agriculture and will not be stimulated to innovate new solutions. Thus, one can quote Confucious: 'If language is incorrect, what is said is not meant. If what is said is not meant, what ought to be done remains undone'. If weeds are not considered a constraint, biotechnological solutions will not be found to deal with them.

Some of the half-baked suggestions made above of what might be considered as possible solutions to weed problems will probably come to naught in the field. Still, if this science fiction suggests better solu-

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tions, the efforts in conceiving them on paper were not in vain.

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Herbicide-Degrading α-Keto Acid-Dependent Enzyme TfdA: Metal Coordination Environment and Mechanistic Insights†

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ABSTRACT: TfdA is a non-heme iron enzyme which catalyzes the first step in the oxidative degradation of the widely used herbicide (2,4-dichlorophenoxy)acetate (2,4-D). Like other α-keto acid-dependent enzymes, TfdA utilizes a mononuclear Fe(II) center to activate O_2 and oxidize substrate concomitant with the oxidative decarboxylation of α-ketoglutarate (α-KG). Spectroscopic analyses of various Cu(II)-substituted and Fe(II)-reconstituted TfdA complexes via electron paramagnetic resonance (EPR), electron spin−echo envelope modulation (ESEEM), and UV−vis spectroscopies have greatly expanded our knowledge of the enzyme's active site. The metal center is coordinated to two histidine residues as indicated by the presence of a five-line pattern in the Cu(II) EPR signal, for which superhyperfine splitting is attributed to two equivalent nitrogen donor atoms from two imidazoles. Furthermore, a comparison of the ESEEM spectra obtained in H_2O and D_2O demonstrates that the metal maintains several solvent-accessible sites, a conclusion corroborated by the increase in multiplicity in the EPR superhyperfine splitting observed in the presence of imidazole. Addition of α-KG to the Cu-containing enzyme leads to displacement of an equatorial water on copper, as determined by ESEEM analysis. Subsequent addition of 2,4-D leads to the loss of a second water molecule, with retention of a third, axially bound water. In contrast to these results, in Fe(II)-reconstituted TfdA, the cosubstrate α-KG chelates to the metal via a C-1 carboxylate oxygen and the α-keto oxygen as revealed by characteristic absorption features in the optical spectrum of Fe−TfdA. This binding mode is maintained in the presence of substrate, although the addition of 2,4-D does alter the metal coordination environment, perhaps by creating an O_2-binding site via solvent displacement. Indeed, loss of solvent to generate an open binding site upon the addition of substrate has also been suggested for the α-keto acid-dependent enzyme clavaminate synthase 2 [Zhou et al. (1998) *J. Am. Chem. Soc. 120*, 13539−13540]. Nitrosyl adducts of various Fe−TfdA complexes have also been investigated by optical and EPR spectroscopy. Of special interest is the tightly bound NO complex of Fe−TfdA·(α-KG)·(2,4-D), which may represent an accurate model of the initial oxygen-bound species.

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α-Keto acid-dependent enzymes are the largest class of mononuclear non-heme iron oxygen-activating enzymes, catalyzing the oxidation of aliphatic C−H bonds in reactions that are even more diverse and varied (*1*−*3*) than those performed by the cytochromes P-450 and methane monooxygenase. The array of reactions catalyzed includes the posttranslational hydroxylation of amino acid side chains during collagen biosynthesis, the conversion of thymine to 5-hydroxymethyluracil in pyrimidine catabolism, the oxidative cyclization of proclavaminate in the biosynthesis of a clinically important β-lactamase inhibitor, and the synthesis of carnitine, a substance critical for both fatty acid anabolism and catabolism (*1*−*3*). The general reaction catalyzed by α-keto acid-dependent enzymes (Scheme 1) involves the oxidative decarboxylation of an α-keto acid cosubstrate, typically α-ketoglutarate (α-KG),[1] concomitant with the oxidation of the primary substrate (*2*). Detailed kinetic data on a number of α-keto acid-dependent enzymes indicate that they utilize an ordered binding mechanism, whereby iron binds first, followed by α-KG, and then either substrate or O_2, the order of which is enzyme dependent (*4*−*7*). The exact

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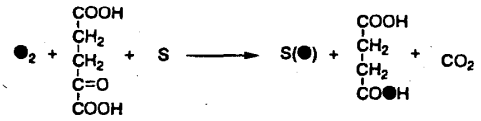
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[1] Abbreviations: CS2, clavaminate synthase 2; 2,4-D, (2,4-dichlorophenoxy)acetate; DAOCS, deacetoxycephalosporin C synthase; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; ESEEM, electron spin−echo envelope modulation; EXAFS, extended X-ray absorption fine structure; fwhm, full width at half-maximum; HPPD, 4-hydroxyphenylpyruvate dioxygenase; IPNS, isopenicillin N synthase; α-KG, α-ketoglutarate; MLCT, metal-to-ligand charge transfer; Mops, 4-morpholinepropanesulfonic acid; TauD, taurine/α-KG dioxygenase; TfdA, (2,4-dichlorophenoxy)acetate/α-KG dioxygenase.

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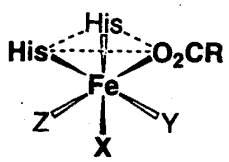
Scheme 1: General Reaction Performed by α-Keto Acid-Dependent Enzymes



mechanism utilized by this class of enzymes, however, is currently unknown.

The crystal structures of two α-keto acid-dependent enzymes have recently been reported. Deacetoxycephalosporin C synthase (DAOCS), which catalyzes the transformation of penicillin N to deacetoxycephalosporin (*8*, *9*), contains a mononuclear iron center ligated to two histidines and an aspartate, while the structure of 4-hydroxyphenylpyruvate dioxygenase (HPPD), an enzyme involved in the catabolism of tyrosine, reveals a mononuclear iron coordinated to two histidines and a glutamate (*10*). These are the only endogenous ligands, which, in both cases, occupy a single face of the polyhedron. Interestingly, the active site of DAOCS and HPPD contain a number of structural features which are common to other crystallographically characterized mononuclear, non-heme iron enzymes including the extradiol ring-cleaving catechol dioxygenases 2,3-dihydroxybiphenyl 1,2-dioxygenase (*11*, *12*), catechol 2,3-dioxygenase (*13*), and protocatechuate 4,5-dioxygenase (*14*), the pterin-dependent tyrosine (*15*) and phenylalanine (*16*) hydroxylases, the Rieske-type naphthalene 1,2-dioxygenase (*17*), and isopenicillin N synthase (IPNS) (*18*). These common features include a "2-His−1-carboxylate facial triad" as the endogenous ligand set and the availability of three cis-oriented coordination sites for the binding of exogenous ligands (*19*) (Figure 1). An attractive hypothesis for the prevalence of this conserved structural motif in non-heme iron oxygen-activating enzymes is that the Fe(II) center has the flexibility to coordinate both substrate and O_2 (*19*). Furthermore, in contrast to heme enzymes, the cis-orientation of the available coordination sites on non-heme iron enzymes may allow intramolecular attack by the reactive oxygen species on the coordinated substrate (*19*).

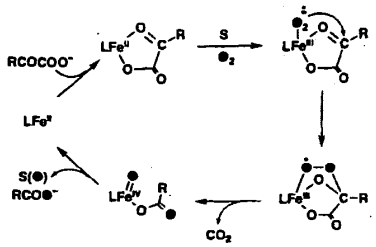
The crystal structure of the DAOCS·(α-KG) complex has also been reported and reveals α-KG to be ligated to the iron center through a C-1 carboxylate oxygen and the α-keto oxygen (*9*). This binding motif, which was anticipated by biomimetic complexes (*20*−*22*) and has recently been proposed for α-KG binding to clavaminate synthase 2 (CS2) (*23*, *24*), maintains one available coordination site on the iron, presumably for coordinating O_2. Binding of O_2 to the Fe(II) center is proposed to generate a metal-coordinated



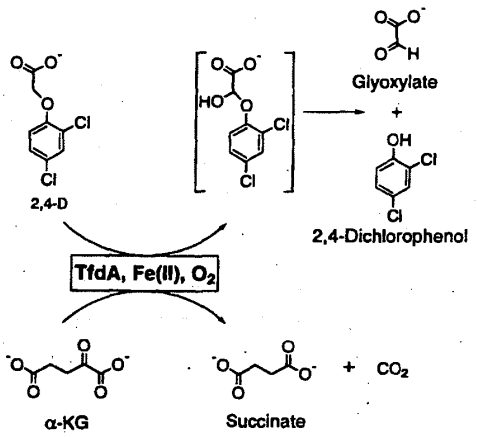
R = Glu or Asp

FIGURE 1: The 2-His-1-carboxylate facial triad utilized by many O_2-activating mononuclear non-heme iron enzymes.

Scheme 2: Proposed Reaction Mechanism for α-Keto Acid-dependent Enzymes



Scheme 3: Reaction Catalyzed by TfdA



superoxide which may then attack the α-carbon of α-KG to initiate oxidative decarboxylation and formation of a putative high valent iron−oxo species (*20*, *21*, *25*, *26*), the species postulated to be responsible for the subsequent oxidation reaction (Scheme 2).

To gain further insight into the iron coordination environment and the mechanism of oxygen activation by α-keto acid-dependent enzymes, we have performed extensive spectroscopic studies on 2,4-dichlorophenoxyacetate/α-KG dioxygenase (TfdA). This enzyme catalyzes the first step in the biodegradation of the widely used herbicide 2,4-dichlorophenoxyacetate (2,4-D), as illustrated in Scheme 3 (*27*, *28*). TfdA possesses a His-X-Asp-$X_{(51)}$-His sequence motif (*29*), much like that found in IPNS and DAOCS; for the latter two enzymes these residues are known to function in metal ligation (*9*, *18*, *30*). While it is tempting to assume from the presence of this motif that TfdA also utilizes these same endogenous ligands, caution is warranted since TfdA displays very limited sequence homology to either of the other two enzymes. DAOCS, on the other hand, exhibits considerable sequence (20.6% identity and 32.2% similarity according to the comparison program GAP) and structural homology with IPNS (*9*). Therefore, understanding the iron coordination environment and mechanism of the herbicide-degrading TfdA has obvious importance not only in the area of bioremediation but also in the general understanding of how this varied and ubiquitous class of enzymes binds and activates dioxygen. We describe herein our spectroscopic studies on TfdA and place these results in perspective with

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other data recently obtained on this class of enzymes (*8*, *9*, *18*, *23*, *24*, *31*, *32*).

EXPERIMENTAL SECTION

Chemicals. Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2$] and Mops were purchased from Sigma (St. Louis, MO), while 2,4-D, $CuCl_2 \cdot 2H_2O$ and the monosodium salt of α-KG were all obtained from Aldrich (Milwaukee, WI). Isotopically pure cupric oxide (^{63}CuO) was purchased from Cambridge Isotope Laboratories (Andover, MA). Argon and nitric oxide gas cylinders were obtained from Airgas, Inc. (Radnor, PA) and Matheson Gas Products, Inc. (Parsippany, NJ), respectively. All solutions were prepared with water, which was purified by passage through a Millipore (Bedford, MA) purification system.

Enzyme Isolation. The homodimeric 64 kDa enzyme TfdA was purified from *Escherichia coli* DH5α cells carrying the *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) JMP134 *tfdA* gene on a pUS311 plasmid as previously described (*27*). TfdA apoprotein was concentrated via centrifugation using Centricon-10 or Centricon-30 concentrators from Amicon. Buffer exchange was accomplished either via repeated concentration/dilution steps or using PD-10 desalting columns from Amersham Pharmacia Biotech (Wikströms, Sweden). Protein concentrations were ascertained using the Bio-Rad (Hercules, CA) assay utilizing bovine serum albumin as a standard. Alternatively, protein concentrations were assessed from the absorbance at 280 nm using a calculated (*33*) ϵ_{280} of 30 600 M^{-1} cm^{-1} per subunit (or 1.0 $AU_{280} \approx 0.96$ mg/mL) based on the amino acid sequence which contains 7 tyrosines, 4 tryptophans, and 13 phenylalanines (*29*).

Activity Assays. The specific activity of TfdA was assessed by reacting the product 2,4-dichlorophenol with 4-aminoantipyrine to give a colored product that was monitored spectrophotometrically (*27*). Alternatively, the activity was assayed by measuring O_2 uptake using a Gilson (Middleton, WI) model K-ICT C Oxygraph containing a 1.5 mL cell equipped with a YSI (Yellow Springs, OH) model 5331 Oxygen Probe, which reduces O_2 to water at the electrode surface; the oxygraph was calibrated by adding concentrated protocatechuate 3,4-dioxygenase to a known quantity of the substrate protocatechuate. Oxygen-uptake reactions were performed with an enzyme concentration of 1−10 μM subunit at 25 °C in 25 mM Mops buffer at pH 6.9. In a typical assay, anaerobic concentrated iron(II)-reconstituted TfdA was added to a stirring aerobic solution containing 66 μM α-KG, 0.33 mM 2,4-D, and 50 μM sodium ascorbate as a reductant.

Preparation of Cu−TfdA Samples. TfdA was reconstituted with copper by the slow addition of an aqueous $CuCl_2$ solution to TfdA apoprotein in 25 mM Mops buffer at pH 6.9 (rapid addition leads to some precipitation of protein). Substoichiometric amounts of copper (0.9 equiv/subunit) were added relative to the enzyme concentration, and electron paramagnetic resonance (EPR) spin quantitation indicated that all of the Cu^{2+} ions were bound to the enzyme. Samples containing α-KG and/or 2,4-D were prepared by the addition of 10 equiv of the appropriate substrate (in 25 mM Mops buffer at pH 6.9) to the Cu−TfdA solution. Typical sample concentrations were 500 μM TfdA subunit, 450 μM Cu^{2+}, 5.0 mM α-KG, and 5.0 mM 2,4-D. In cases where exogenous imidazole was added to the solution, the imidazole/copper ratio was maintained at 15. Isotopically pure [^{63}Cu]TfdA samples were prepared in an identical fashion except that $^{63}CuCl_2$ was prepared from ^{63}CuO by dissolving the cupric oxide in a minimal volume of concentrated HCl, then diluted with H_2O and adjusted to approximately pH 4 using NaOH(aq), yielding a final $^{63}CuCl_2$ stock concentration of 12 mM. Dual samples for electron spin−echo envelope modulation (ESEEM) spectroscopy were prepared in H_2O/D_2O at pH/pD 7.0 using $CuCl_2$ as described above except that the concentration of buffer was 50 mM and only 5 equiv of α-KG and 2,4-D were utilized.

Preparation of Fe−TfdA Samples. All samples were prepared anaerobically in an ice bath to prevent enzyme turnover, oxidative inactivation (*34*), and autoxidation of Fe^{2+}. TfdA solutions were made anaerobic by placing a small volume of concentrated TfdA (approximately 50−100 μL in 100 mM Mops buffer, pH 6.9 at 4 °C) in a heart-shaped Schlenk flask followed by two rapid vacuum/argon cycles. The enzyme was then allowed to equilibrate under Ar while stirring for approximately 15 min, followed by an additional 15 min of stirring under an Ar stream. All other solutions were prepared using standard Schlenk vacuum line techniques and contained 100 mM Mops buffer, pH 6.9, at 4 °C. TfdA apoprotein was reconstituted by the addition of a substoichiometric quantity of iron (0.8−0.9 equiv) from an ice-cold $Fe(NH_4)_2(SO_4)_2$ solution using gastight Hamilton syringes (Reno, NV). Samples containing α-KG and/or 2,4-D (5.0 equiv) were prepared by anaerobic addition of a concentrated buffered solution of the appropriate substrate to the Fe−TfdA solution. Typical TfdA concentrations ranged from 0.3 to 0.6 mM subunit for optical spectroscopy and 0.1 to 0.4 mM for EPR spectroscopy.

Preparation of NO Samples. Fe−TfdA solutions were briefly incubated with nitric oxide (NO) gas in either a Schlenk flask or Schlenk cuvette via headspace diffusion using standard Schlenk vacuum line techniques. The solutions were gently mixed until the samples exhibited the brillant yellow color characteristic of iron−nitrosyl complexes. Excess NO was removed by vacuum. To ensure that adventitous nitric acid was not introduced into the sample, the gas line was equipped with a column of NaOH pellets to "scrub" the NO and the line was purged with Ar for 30 min before using. ***Caution***: NO is a poisonous gas and should only be used with proper ventilation.

Electron Paramagnetic Resonance (EPR) Spectroscopy. X-band EPR spectra were recorded using either a Varian E−109 spectrometer or a Bruker E-500 spectrometer, both of which were equipped with an Oxford Instruments ESR-10 liquid helium cryostat. Samples were placed into 0.3 mm i.d. quartz tubes and frozen in liquid nitrogen. Cu−TfdA spectra were acquired at 20 K using a power of 0.20 mW. In general, the spectra were obtained as 4.0 min scans from 2200 to 2800 G using a time constant of 0.128 s, a modulation amplitude of 10 G, and a modulation frequency of 100 kHz. To obtain high-resolution spectra of the farthest downfield $g_{||}$ hyperfine line of Cu−TfdA, 4.0 min scans were obtained from 2560 to 2640 G using a time constant of 2.0 s, a modulation amplitude of 2.0 G, and a modulation

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frequency of 100 kHz. The high-resolution EPR spectra were fit using the program QPOWA (*35*). Nitrosyl Fe−TfdA spectra were collected as 168 s scans at 8.0 K from 0 G to 5000G at a power of 0.20 mW using a time constant of 0.164 s, a modulation amplitude of 10 G, and a modulation frequency of 100 kHz. Spin quantification of both Cu−TfdA and Fe−TfdA samples was performed by integrating the EPR absorption spectra and comparing the results with that of an aqueous CuEDTA solution of known concentration (*36*). In performing the spin quantifications, appropriate corrections were made for different EPR parameters between the standard and samples. In all cases, power saturation studies were performed to ensure that the signals were not saturated under the conditions utilized.

Electron Spin−Echo Envelope Modulation (ESEEM) Spectroscopy. Pulsed EPR data were collected on a home-built spectrometer as previously described (*37*). ESEEM data were collected in the usual point-by-point fashion using a Tektronix 620 B digital oscilloscope to perform signal averaging and numerical integration of the echo amplitudes. Data acquisition was controlled by a Power Computing model 200 computer, using software created with the LabView, version 5.0, programming package (National instruments). The microwave probe utilized a folded stripline resonant element (*38*) and a Gordon coupling scheme suitable for liquid helium immersion experiments (*39*). For two-pulse ESEEM experiments (90°−τ−180°), τ was varied from 150 ns to ~2.5 μs for each data set containing 512 points. Spectra were collected at 4.2 K using a microwave pulse power of 20 W, a pulse width of 20 ns (fwhm), and a repetition rate of 40 Hz. The magnetic field strength was set to 3050 G with a microwave frequency of approximately 8.8 GHz. Data analysis was performed using Matlab version 5.2 (Mathworks); dead time reconstruction (*40*) was used to process the data for the ESEEM envelopes prior to Fourier transformation. Computer simulations of deuterium ESEEM spectra were done using software based on the density matrix treatment of Mims (*41*).

Electronic Absorption Spectroscopy. Anaerobic Fe−TfdA samples were prepared as described above and placed in a 0.8 mL dual path length (2.0 mm × 1.0 cm) quartz cuvette (Wilmad Glass; Buena, NJ) which had been modified to contain a Schlenk-type fitting. Spectra were recorded on a scanning Beckman DU 640 spectrophotometer equipped with a refrigerated circulating bath. Typical enzyme concentrations (expressed as the number of active sites) were 0.3−0.6 mM. The reference sample contained only 100 mM Mops buffer. Spectra were obtained at 4 °C to stabilize the enzyme.

RESULTS

EPR Studies of Cu(II)-Substituted TfdA. To provide a means to probe the spectroscopically inaccessible Fe(II) active site and to circumvent the oxygen reactivity of Fe−TfdA, we replaced the native Fe(II) ion with Cu(II), an ion that possesses rich EPR properties and whose coordination chemistry is well characterized (*42*). Significantly, Cu(II) is a very strong competitive inhibitor of TfdA activity (K_i = 0.1−0.3 μM) with respect to Fe(II), suggesting that the Cu(II) ion replaces Fe(II) at the active site. In fact, the K_i of Cu(II) is significantly lower than the concentration of Fe(II) required for TfdA to achieve half-maximal activity (10−30 μM). Thus, while Cu(II) coordination is unlikely to exactly model that of Fe(II) due to the preference of Cu(II) for tetragonal geometries as opposed to the octahedral geometry common for Fe(II), analysis of the TfdA-bound copper environment is likely to reflect the important features of the native TfdA metallocenter.

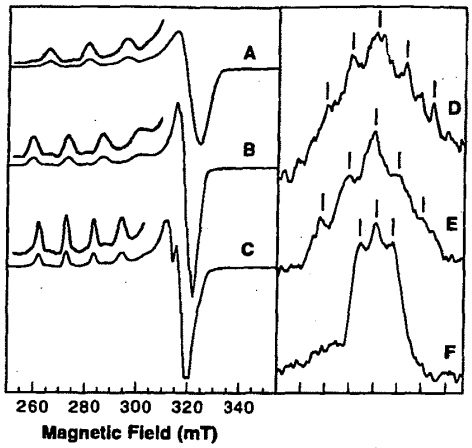


FIGURE 2: EPR spectra of Cu−TfdA complexes. (A) Cu−TfdA ($g_\perp$ = 2.07, $g_\parallel$ = 2.29, $A_\parallel$ = 16.1 mK, 4.2 mT fwhm at 281 mT); (B) Cu−TfdA·(α-KG) ($g_\perp$ = 2.07, $g_\parallel$ = 2.36, $A_\parallel$ = 14.9 mK, 4.2 mT fwhm at 273 mT); (C) Cu−TfdA·(α-KG)·(2,4-D) (g_x = 2.07, g_y = 2.11, g_z = 2.37, A_z = 12.0 mK, 2.2 mT fwhm at 273 mT). Insets show $g_\parallel$ with the intensity magnified 5 times. Spectra D−F display superhyperfine splitting of the $g_\parallel$ region in [^{63}Cu]TfdA EPR spectra due to ^{14}N-containing (I = 1) ligands. While the spectra utilize different x-axes in order to align the peaks, each tick mark represents 1 mT for all three spectra. (D) [^{63}Cu]TfdA; (E) [^{63}Cu]-TfdA·(α-KG); (F) [^{63}Cu]TfdA·(α-KG)·(2,4-D). Spectra D−F were simulated with QPOWA (*33*) as described in the Experimental Section. Spectra D and E display 5-fold splitting (A = 1.0 mT) while spectrum F displays 3-fold splitting (A = 0.7 mT).

The EPR spectra of various copper-substituted TfdA complexes were obtained and have provided considerable insight into the nature of the endogenous ligands coordinated to the metal in the active site (*32*). In the absence of either the cosubstrate α-KG or the primary substrate 2,4-D (Figure 2A), Cu−TfdA exhibits an axial spectrum with g- and A-values ($g_\perp$ = 2.07; $g_\parallel$ = 2.29; $A_\parallel$ = 16.1 mK)[2] consistent with the Cu(II) ion possessing tetragonal geometry (*43*) and a ligation environment containing a mixture of N and O donors (*44*). This EPR spectrum is perturbed by the addition of the cosubstrate α-KG (Figure 2B), with $g_\perp$ = 2.07, $g_\parallel$ = 2.36, and $A_\parallel$ = 14.9 mK. While the addition of 2,4-D to Cu−TfdA has no effect on the EPR spectrum in the absence of α-KG, addition of 2,4-D to the binary Cu−TfdA·(α-KG) complex results in a more rhombic signal and a considerable sharpening of the spectrum from 4.2 mT (fwhm at 273 mT) to 2.2 mT (fwhm at 273 mT) with g_x = 2.07, g_y = 2.11, g_z = 2.37, and A_z = 12.0 mK (Figure 2C). A comparison of the three spectra shows a progressive movement to lower field for $g_\parallel$ and a decrease in the $A_\parallel$ values in the binary and ternary complexes. This is a significant trend since an

[2] A keiser (K) is equivalent in energy to a reciprocal centimeter (cm^{-1}). Therefore, using the units of K, $A_\parallel$ = (g-value) × (splitting in gauss) × (β_e).

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increase in $g_{||}$ is usually indicative of a shift to a more oxyanion-rich environment (*44*), while small $A_{||}$ values suggest a significant distortion from planarity (*45*, *46*). For example, the $g_{||}$ and $A_{||}$ values of the ternary Cu−TfdA·(α-KG)·(2,4-D) complex are comparable to those of Cu-substituted ribulose-1,5-bisphosphate carboxylase/oxygense complexed to the transition-state analogue 2-carboxyarabinitol bisphosphate [$g_{||} = 2.41$, $A_{||} = 11.59$ mK (*47*)] and Cu(II)-substituted enolase complexed to 2-phosphoglycerate [$g_{||} = 2.40$, $A_{||} = 12.0$ mK (*48*)], both with crystallographically determined structures which reveal an oxyanion-rich environment at a distorted metal center (*49*, *50*).

In a previous communication (*32*), we incorrectly reported that the EPR spectra of Cu−TfdA·(pyruvate) complexes were identical to those with α-KG. This observation led to the erroneous conclusion that the C-5 carboxylate was not important in eliciting the characteristic spectrum of the Cu−TfdA·(α-KG) species. We have since discovered, however, that the pyruvate utilized in the experiments contained small quantities of a "pyruvate dimer" (2-hydroxy-2-methyl-4-oxoglutaric acid), which mimics α-KG in possessing the second carboxyl functional group. On the basis of its structural similarity to α-KG ($K_m = 3.2$ μM), this "dimer" is presumed to have a much lower K_m than pyruvate ($K_m =$ 1.0 mM). Experiments using authentic "pyruvate dimer" (synthesized by treating pyruvate with concentrated NaOH for at least 30 min) yielded the EPR spectrum characteristic of the α-KG-bound state, whereas those using ultrapure pyruvate failed to reproduce this spectroscopic feature. Thus, the C-5 carboxylate is, in fact, important in the binding of α-KG despite the fact that this carboxylate presumably does not coordinate to the metal center.

Further information about the coordination environment of the metal center was obtained from analysis of the superhyperfine splitting in the EPR spectra. This phenomenon arises from the interaction of the unpaired electron on the Cu(II) ion with the nuclear spin (I) of an atom directly bound to the metal and lying in the principal tetragonal plane (superhyperfine splitting is not observed for axial ligands). In an ideal situation, one can determine the number of nitrogen-containing ligands (while ^{16}O does not have a nuclear spin, ^{14}N has $I = 1$) since the number of superhyperfine lines is equal to $2nI + 1$, where n is the number of equivalent nuclei. An excellent example of such an analysis of superhyperfine splitting was recently reported by Ballou and co-workers in their study of Cu(II)-substituted phthalate dioxygenase (*51*).

Although not readily apparent from Figure 2, superhyperfine splitting is present in the $g_\perp$ region of the Cu−TfdA EPR spectra, and this splitting can be discerned in the second derivative spectra. Figure 3A shows the EPR second derivative spectrum of Cu−TfdA in the $g_\perp$ region. Unfortunately, the resolution of ligand superhyperfine features is poor, indicating that the structure about the metal center may be somewhat heterogeneous prior to substrate addition. When α-KG binds to the holoenzyme, however, the resolution of the superhyperfine features is greatly enhanced, and an apparent 1:2:3:2:1 quintet is observed (Figure 3C), consistent with two nitrogen-containing endogenous ligands. Enhanced resolution of the ligand superhyperfine features at $g_\perp$ is also observed in the presence of a 15-fold excess of imidazole (Figure 3B). The appearance of at least nine features in this

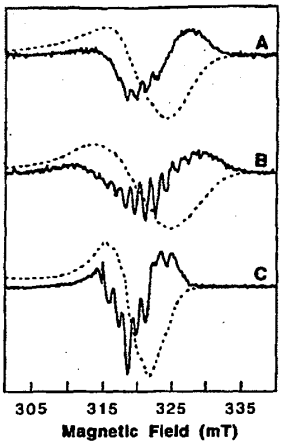


FIGURE 3: Superhyperfine splitting of the $g_\perp$ region in Cu−TfdA EPR spectra due to ^{14}N-containing ($I = 1$) ligands. (A) Cu−TfdA; (B) Cu−TfdA in the presence of 15 equivalents of imidazole; (C) Cu−TfdA·(α-KG). The dashed lines are the expanded first derivative $g_\perp$ signals, while the solid lines are the second derivative $g_\perp$ signal in which the superhyperfine splitting is more easily observed.

spectrum is commensurate with the addition of 1−2 exogenous imidazole ligands to the Cu(II) tetragonal plane, in addition to the two derived from the protein (*32*). These results suggest that TfdA maintains solvent-accessible sites on the metal ion which are presumably available for the binding of exogenous ligands.

The breadth of the $g_\perp$ feature as shown in the second derivative spectra of Figure 3, however, indicates that $A_\perp^{Cu}$ is similar in magnitude to the nitrogen superhyperfine splitting of the equatorially bound histidyl or imidazole ligands. This renders an analysis of the number of nitrogen-based ligands based on counting superhyperfine features at $g_\perp$ tenuous and led us to a detailed examination of ligand superhyperfine structure in the $g_{||}$ region. Splitting in this region is often difficult to resolve because natural abundance copper contains a mixture of ^{63}Cu (69%) and ^{65}Cu (31%), both of which are $I = 3/2$ nuclei but give rise to slightly different $A_{||}$ values. To increase the likelihood of observing the superhyperfine splitting, isotopically pure Cu(II) was utilized. The farthest downfield $g_{||}$ signal of samples prepared with [^{63}Cu]TfdA (Figure 2) exhibits improved resolution relative to that obtained using natural abundance copper and provides information complementary to that derived from the $g_\perp$ region. For Cu−TfdA, a 1:2:3:2:1 pattern expected from two equivalent nitrogens may be discerned (Figure 2D). This five-line pattern is more clearly resolved in the Cu−TfdA·(α-KG) complex (Figure 2E), supporting the coordination of two nitrogen-containing ligands to the metal center. In the presence of both α-KG and 2,4-D (Figure 2F), however, the EPR spectrum displays only 3-fold splitting, indicating a loss of one of the nitrogen ligands from the tetragonal plane of Cu(II). These results suggest that 2,4-D binding either displaces one of the His ligands or causes a reorientation of the principal tetragonal plane of the Cu(II) ion such that one of the nitrogen ligands shifts to an axial position.

ESEEM Spectroscopy of Cu(II)-Substituted TfdA. To gain further information on the number and identity of the

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metallocenter ligands, Cu−TfdA samples were also studied by the ESEEM method of pulsed EPR spectroscopy. Previous ESEEM studies on this system provided evidence for the equatorial ligation of two His ligands for Cu−TfdA and the Cu−TfdA·(α-KG) adduct (*32*), in agreement with the results of continuous wave EPR experiments presented above. The ESEEM from one of these His ligands was lost upon the addition of 2,4-D to the Cu−TfdA·(α-KG) complex, consistent with the observed decrease in the multiplicity of the superhyperfine splitting in the ternary Cu−TfdA·(α-KG)·(2,4-D) complex. Because the model for Fe(II) coordination in this class of enzymes calls for the coordination of multiple water ligands that are displaced as the cosubstrates bind to the metal, ESEEM experiments were performed on samples exchanged against D_2O. The overall strategy for these measurements makes use of the product rule for ESEEM, which states that the modulation function for a discrete paramagnetic center consists of a product of a background decay function, governed by spin relaxation processes, with the individual modulation functions due to all of the coupled nuclei (*52*, *53*). If ESEEM data collected for samples exchanged against D_2O-based buffer are divided by ESEEM data collected under identical conditions for like samples in aqueous buffer, the resulting modulation function will contain only contributions arising from exchangeable deuterons that are magnetically coupled to the paramagnet (*54*). This technique thus provides a method to study coordinated water molecules in the presence of substantial ESEEM from the remote nitrogens of the coordinated histidyl ligands (*55*).

ESEEM ratios taken for Cu−TfdA, Cu−TfdA·(α-KG), and Cu−TfdA·(α-KG)·(2,4-D) are shown as solid lines in panels A−C of Figure 4, respectively. These data were collected using a two-pulse (90°−τ−180°−τ−echo) microwave excitation scheme because this method provides the most reliable measure of amplitudes for dividing data sets (*54*). Also, the two-pulse data feature a sum combination frequency component that provides an "internal" line width, or damping standard for distinguishing bound D_2O molecules from ambient waters (*55*). For all three enzyme samples, the data of Figure 4 show intense modulations that consist of major frequency components at 2.0 MHz (modulation period = 500 ns), the Larmor frequency of deuterium in a 0.305 T magnetic field, and a sum combination frequency centered at 4.1 MHz (modulation period ≈ 250 ns). In agreement with the proposal that the cosubstrates displace water ligands when they bind to Cu(II), the depth, or amplitude, of the modulations decreases as one goes from the Cu−TfdA data (Figure 4A) to the data for the Cu−TfdA·(α-KG)·(2,4-D) ternary complex (Figure 4C). Some caution needs to be taken when considering these results, however, since the ^{2}H-ESEEM shown in Figure 4 originates from D_2O molecules bound to the metal, ambient D_2O molecules close enough to the Cu(II) to magnetically couple to it, and deuterons that have exchanged with the exchangeable protons of TfdA near the metal site.

A useful means to differentiate the different populations of deuterons that contribute to the ESEEM is to make use of the relative amplitudes of the ^{2}H fundamental and sum combination frequency components. Figure 5 shows two-pulse ESEEM simulations for D_2O molecules bound to Cu(II) at an equatorial position (trace A) and an axial position (trace B). Figure 5C shows the predicted two pulse ESEEM

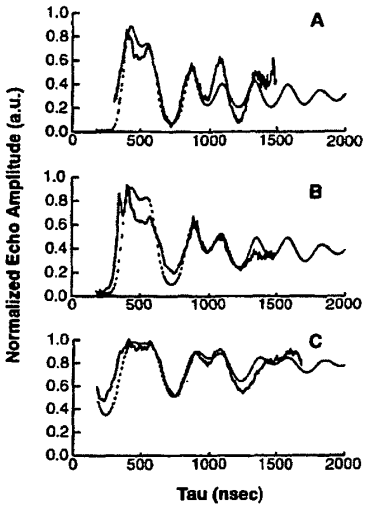


FIGURE 4: The solid lines are deuterium ESEEM data collected for (A) Cu−TfdA, (B) Cu−TfdA·(α-KG), and (C) Cu−TfdA·(α-KG)·(2,4-D). These data were obtained by dividing the two-pulse ESEEM data collected for each sample exchanged against D_2O-based buffer with the identical sample in H_2O-based buffer. Prior to division, each data set was normalized so that the ordinate ranged 0−1, where 0 amplitude was measured directly for each scan and 1 was assigned to the maximum amplitude found experimentally. Conditions common to these measurements were microwave frequency, 8.78 GHz; magnetic field strength, 0.305 T; microwave pulse power, 25 W; pulse width, 20 ns (fwhm); pulse sequence repetition rate, 40 Hz; 40 events were averaged at each time point and four complete scans were averaged to arrive at each ESEEM trace. The dashed lines are ESEEM simulations for (A) two equatorial and one axial bound D_2O molecules, (B) one equatorial and one axial bound D_2O, and (C) one axially bound D_2O. Simulations used the Hamiltonian parameters for equatorial and axial bound D_2O molecules given in the captions for Figure 5, respectively.

for a D_2O molecule with both deuterons positioned 4 Å away from the Cu(II) and coupled by a dipolar, or through-space mechanism. The hyperfine splitting constants used for these simulations were taken from a single-crystal ENDOR study of Cu(II)$(H_2O)_6$ in Tutton salts and are given in the figure caption (*56*). Figure 5A shows a deuterium ESEEM pattern that features intense modulation at the deuterium Larmor frequency (2.0 MHz) which damps quickly and leaves only modulations at the sum combination frequency (4.1 MHz) at longer times. The axially bound D_2O (Figure 5B) shows deep modulations, but the fundamental frequency is less damped than it was for the equatorial case so that the second harmonic is less prominent at longer values of τ. The ambient water shows modulations that are a factor of 7−8 less intense than those of the bound waters with a substantially reduced contribution of the sum combination frequency (Figure 5C). These effects are conveniently viewed in the frequency domain as shown in Figure 6 where Fourier transforms of the simulations for equatorial (Figure 6A), axial (Figure 6B), and ambient (Figure 6C) D_2O molecules are provided. For equatorially bound D_2O, the anisotropic contribution to the hyperfine splitting is large (the effective dipole−dipole distance is about 2.4 Å), and that serves to broaden the width of the 2.0 MHz peak. Because the deuterium hyperfine splitting is of opposite sign in the two-electron spin manifolds

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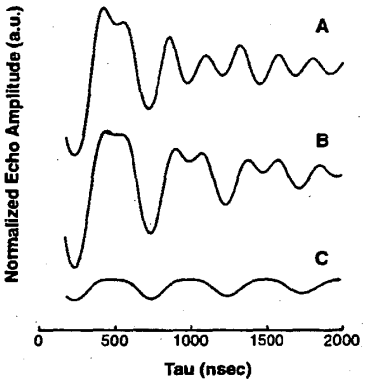


FIGURE 5: ^{2}H-ESEEM simulations for (A) a D_2O molecule bound equatorially to Cu(II), (B) an axially bound D_2O, and (C) a D_2O molecule coupled through space to Cu(II) so that the deuterium–copper distance was 4 Å. Hamiltonian parameters for the ESEEM simulations were for equatorially bound D_2O; $A_{xx} = -1.4$ MHz, $A_{yy} = -0.6$ MHz, $A_{zz} = 1.4$ MHz, nuclear g-value = 0.857 44, magnetic field = 0.305 T, starting $\tau = 180$ ns, $e^2qQ = 0.2$ MHz, nqi asymmetry parameter = 0.5 and the nqi and hyperfine tensors were taken as coaxial. Parameters were the same for the axially bound D_2O except for the hyperfine splitting, $A_{xx} = -0.5$ MHz, $A_{yy} = -0.5$ MHz, and $A_{zz} = 1.1$ MHz. For ambient D_2O (trace C), $A_{xx} = -0.2$ MHz, $A_{yy} = -0.2$ MHz, and $A_{zz} = 0.4$ MHz.

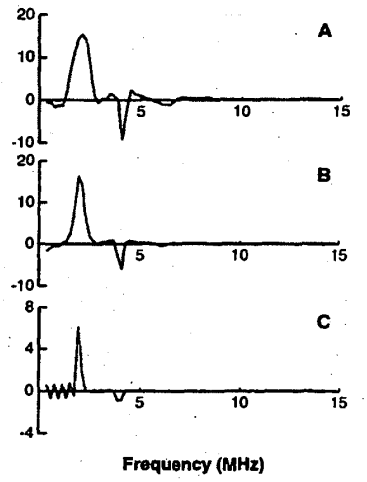


FIGURE 6: Fourier transforms of the ESEEM simulations shown in Figure 5. Trace A is for an equatorially bound D_2O, trace B an axially bound D_2O, and trace C ambient D_2O.

that combine to yield the sum combination peak, the width of the 4.1 MHz line remains narrow for all three of the splitting cases depicted in Figures 5 and 6. This feature is broadened only by the small nuclear quadrupole interaction that is intrinsic to the D_2O molecule.

The simulations of Figures 5 and 6 suggest that a simple method to gauge the relative contributions of equatorial and axial water coordination to Cu(II) ESEEM data is to determine the ratio of the amplitudes of first and second harmonics of the deuterium modulation frequencies as measured from the ESEEM spectra. For the simulation of equatorially bound D_2O (Figures 5A and 6A), this ratio is 1.5; for axially bound D_2O (Figures 5B and 6B), the 2/4 MHz intensity ratio climbs to 2.5; and for ambient water at 4 Å, a ratio of >5 is found. Table 1 shows the results of determining these ratios for simulations carried out for several cases of mixed equatorial/axial D_2O ligation. It is apparent that if both equatorial and axial D_2O ligands are present, the 2/4 MHz amplitude ratio is close to 2.0, a value that is intermediate to those representing pure axial or equatorial ligation. The chief source of errors in measuring peak amplitudes and computing these ratios comes from the reduced frequency resolution realized for 2-pulse ESEEM and difficulties in estimating the position of the baseline for each peak. The sum combination frequency can be measured more reliably because of its narrow line width and negative phase. In contrast, it is often difficult to estimate the baseline position for the low ^{2}H fundamental frequency because the baseline "roll", which stems from shortcomings of the dead-time reconstruction procedure (*40*), is most severe near "0" MHz. The error for computing these peak ratios from the FT is probably ±0.1, for the simulations, and ± 0.2, for the experimental data of Figure 4.

Table 1: Ratios of ^{2}H-ESEEM Fundamental (2 MHz) and Sum-Combination (4 MHz) Frequencies for Various D_2O Coordination Schemes

(A) Computer Simulations

coordinated D_2O molecules	2/4 MHz ratio
2 equatorial, 0 axial	1.5 (±0.1)
1 equatorial, 0 axial	1.5
2 equatorial, 1 axial	1.7
1 equatorial, 1 axial	1.7
2 equatorial, 2 axial	2.0
1 equatorial, 2 axial	2.0
0 equatorial, 1 axial	2.5
0 equatorial, 2 axial	2.5

(B) TfdA Data

sample	2/4 MHz ratio
Cu-TfdA	1.7 (±0.2)
Cu-TfdA·(α-KG)	1.8
Cu-TfdA·(α-KG)·(2,4-D)	2.5

The 2/4 MHz amplitude ratios measured from Fourier analysis of the three TfdA ^{2}H-ESEEM traces of Figure 4 are also listed in Table 1. The ternary complex Cu–TfdA·(α-KG)·(2,4-D) gives a ratio of 2.5, indicating that it has at least one axially bound D_2O ligand and no equatorial waters. For the other two complexes, Cu–TfdA and Cu–TfdA·(α-KG), more ^{2}H second harmonic is observed with these 2/4 MHz peak ratios being 1.7 and 1.8, respectively. These results favor a model for Cu(II) water coordination that begins with two equatorial waters and one axial water for the protein in the absence of the cosubstrates (Cu–TfdA). The addition of α-KG displaces one of the equatorial water molecules to leave one equatorial and one axial water. Finally, formation of the ternary complex leads to the displacement of the remaining equatorial water to yield a Cu(II) site with a single, axially bound water molecule. To test this idea, ^{2}H-ESEEM calculations were carried out using the model simulations of Figure 5, curves A and B, together with the spherical model approximation to the ESEEM product rule (*57*) to produce simulations of the two-equatorial/one-axial, one-equatorial/one-axial, and one-axial cases. The results are superimposed on the data of panels A–C of Figure 4, respectively (dashed lines) and show that

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there is good agreement between the measured ^{2}H-ESEEM functions (solid lines) and the amplitudes predicted theoretically. No attempt was made to adjust the hyperfine parameters from the values determined in the single-crystal ENDOR studies of Cu(II)$(H_2O)_6$ in Tutton salts (*56*).

There are several features of the ^{2}H-ESEEM data shown in Figure 4 that need to be kept in mind when judging the quality of the simulations. These data were obtained by dividing two sets of 2-pulse ESEEM data that featured deep ^{14}N modulations from His coordination and background decay functions due to electron spin−spin relaxation that left only small amplitudes at longer τ. As a result, one is forced to take the ratios of small numbers adding considerable noise to the result that gets worse at longer times. The data shown in Figure 4 are truncated at 1500 ns because the noise in the ratio becomes too severe at higher τ values. The agreement between experiment and theory is worst for the Cu−TfdA sample (solid line of Figure 4A) because the deeper ^{2}H modulation that results from the additional bound water molecule leads to greater uncertainty in the ratio, and additional ambient water, that likely has access to the site when the cosubstrates are absent, is not being considered in the calculation. As a result of these problems, emphasis was placed on comparison between experiment and theory for the first 1 μs of the ^{2}H-ESEEM function of Figure 4A, providing strong evidence that there are two equatorial and one axial D_2O molecules bound to the Cu(II) of TfdA in the absence of the cosubstrates.

In principle, further verification of our ^{2}H-ESEEM modeling of water coordination to Cu−TfdA could be obtained by taking another set of ESEEM ratios. By dividing the ratio of Figure 4A with that of Figure 4B, it should be possible to obtain the ^{2}H-ESEEM pattern of the water displaced by α-KG binding. In a similar fashion, division of Figure 4B by Figure 4C should reveal the ^{2}H-ESEEM function of the D_2O molecule displaced by 2,4-D addition to the protein. Unfortunately, this procedure is only accurate when the continuous wave EPR spectrum remains unchanged as a result of substrate addition. Because there are large changes that occur when adding α-KG to Cu−TfdA, particularly with respect to narrowing the distribution of coordination environments that likely exists for the holo-enzyme, the ratio of trace B in Figure 4 with trace A gave a deep ^{2}H-ESEEM pattern with a 2/4 MHz ratio of 1.1 and an appearance that was much different from the model calculations of Figure 5, traces A and B (data not shown). However, the changes in the EPR spectrum that occur when 2,4-D is added to the Cu−TfdA·(α-KG) complex are more subtle, and a good ratio was obtained by dividing the ^{2}H-ESEEM data of Figure 4, trace B, with that of trace C. The result provides the ^{2}H-ESEEM spectrum of the D_2O displaced by 2,4-D addition and is shown in Figure 7 (solid line). The dashed line superimposed on these data is from the model calculation of the ^{2}H-ESEEM for a D_2O molecule bound equatorially to Cu(II).

Binding of α-KG and 2,4-D to Fe(II)-Reconstituted TfdA. Binding of α-KG to the metal center was assessed directly via optical spectroscopy. In the absence of α-KG, the optical spectrum of Fe−TfdA does not exhibit any absorption features in the visible region, while the UV region is dominated by protein $\pi-\pi^*$ transitions. Lack of a visible chromophore is typical of high-spin Fe(II) species. Upon the anaerobic addition of α-KG, however, the enzyme solution

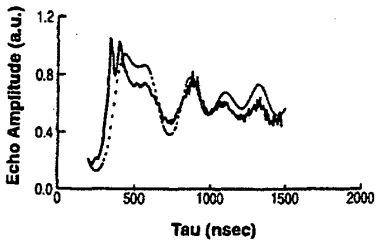


FIGURE 7: The solid line is a ^{2}H-ESEEM trace obtained by dividing the experimental ratios for Figure 4B by that of Figure 4C. The dashed curve is a simulation for a single D_2O molecule bound equatorially to Cu(II) and was obtained by truncating the trace of Figure 5A at 1.5 μs.

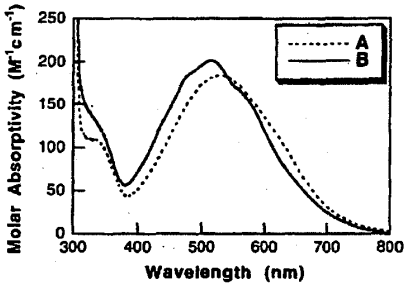


FIGURE 8: Optical absorption spectra of (A) Fe−TfdA·(α-KG) (λ_{max} = 530 nm) and (B) Fe−TfdA·(α-KG)·(2,4-D) (λ_{max} = 515 nm with shoulders at approximately 475 and 560 nm). The spectrum of the Fe−TfdA sample has been subtracted to minimize protein scattering effects.

becomes pinkish and a broad absorption is observed in the visible region centered at approximately 530 nm (Figure 8). Analogous spectra are obtained from synthetic Fe(II)·(α-keto acid) complexes (*20*−*22*, *25*) where the energy of this spectral feature is easily tuned by varying the substituent on the α-keto acid. Electron-donating groups shift the transition to shorter wavelengths (higher energy), while electron-withdrawing groups shift it to longer wavelengths (lower energy) (*20*, *21*), consistent with its assignment as a metal-to-ligand charge transfer (MLCT) transition from the Fe(II) center to the α-keto acid. Significantly, this feature is only observed when the α-keto acid chelates via the carboxylate oxygen and the α-keto oxygen; when the α-keto acid binds in a monodentate fashion, the model iron complexes remain colorless (*21*, *22*). Thus, this broad absorption feature is diagnostic of an α-keto acid chelated to an Fe(II) metal center, indicating that α-KG chelates to the iron in TfdA.

The MLCT transition observed in the presence of α-KG is a convenient probe for monitoring the binding of the primary substrate 2,4-D. As shown in Figure 8, upon the addition of 2,4-D, the absorption is blue-shifted and the optical spectrum sharpens significantly, revealing the presence of at least three different charge-transfer transitions (λ_{max} = 515 nm with shoulders at approximately 475 and 560 nm). These results indicate that α-KG remains chelated to the Fe(II) center in the presence of substrate [i.e., in the Fe−TfdA·(α-KG)·(2,4-D) complex], and that 2,4-D binding affects the coordination environment of the iron(II) center.

Binding of NO to Fe(II)-Reconstituted TfdA. In an effort to gain insight into O_2 binding at the active site, various

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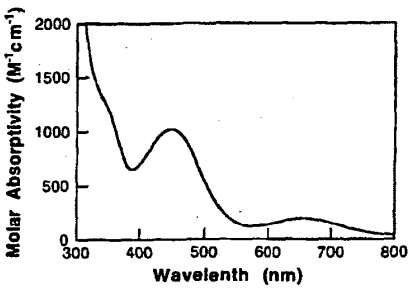


FIGURE 9: Optical absorption spectrum of Fe−TfdA·(α-KG)·(2,4-D)·(NO) (λ_{max} = 449 and 657 nm).

complexes of TfdA were incubated with the O_2 analogue NO. Such an approach has been applied successfully to both model complexes and other iron(II) oxygenases (*58*−*61*). Nitric oxide has an advantage over other O_2-mimics (such as azide) because the radical NO converts the usually nonchromophoric and effectively EPR-silent high-spin (S = 2) Fe(II) ion into an $\{FeNO\}^7$ system (*62*), which is EPR active (S = 3/2) and contains a visible chromophore. Thus, the use of NO provides additional spectroscopic techniques with which to probe the metal center.

Nitric oxide reacts readily with Fe−TfdA, the binary complex Fe−TfdA·(α-KG), and the ternary complex Fe−TfdA·(α-KG)·(2,4-D), as assessed by the formation of characteristic bright yellow iron-nitrosyl species (*58*, *63*). The electronic structure of these complexes appears to be dominated by Fe−NO interactions, since the spectra exhibit only slight variations in the presence/absence of α-KG and 2,4-D [only the Fe−TfdA·(α-KG)·(2,4-D)·(NO) spectrum is shown in Figure 9]. On the basis of detailed spectroscopic analysis of small inorganic Fe−NO model complexes, the absorptions observed at 449 and 657 nm have been assigned by Solomon and co-workers to $NO^- \rightarrow Fe^{3+}$ and Fe^{3+} ligand field transitions, respectively (*63*). The assignment of the TfdA complexes as Fe−NO species was corroborated by EPR spectroscopy (Figure 10), which yielded signals very close to g = 4, 4, and 2 as expected for axial S = 3/2 systems (*58*). The spectra of Fe−TfdA·(NO) and Fe−TfdA·(α-KG)·(NO) are essentially identical with g_x = 4.09, g_y = 3.98, and g_z = 2.00 and a peak-to-peak width of 6.4 mT in the g = 4 region. In the presence of both α-KG and 2,4-D, however, the $\{Fe-NO\}^7$ center becomes more rhombic, with signals at g_x = 4.12, g_y = 3.96, and g_z = 2.00 and a peak-to-peak width of 8.9 mT. This change is mirrored in the copper EPR data of the ternary Cu−TfdA·(α-KG)·(2,4-D) complex which indicates that the copper ion also is in a more rhombic environment. The binding of NO directly to the ternary Fe−TfdA·(α-KG)·(2,4-D) complex indicates that even in the presence of both α-KG and 2,4-D, an available coordination site is maintained on the iron atom for the binding of NO and, by analogy, O_2. In addition, the fact that NO binds directly to the metal ion implies that O_2 activation occurs via direct ligation of O_2 to the Fe(II) center, not via some outer-sphere electron-transfer pathway. The Fe−TfdA·(α-KG)·(2,4-D)·(NO) complex is the best model to date of the active quaternary Fe−TfdA·(α-KG)·(2,4-D)·(O_2) complex, i.e., the species which ultimately gives rise to catalysis.

NO binding appears to be reversible under certain circumstances as evidenced by the frequent presence of free NO in the EPR spectra of both Fe−TfdA·(NO) and Fe−TfdA·(α-KG)·(NO). To further characterize the reversibility of NO binding, optical spectra of various nitrosyl complexes were monitored as the complexes were placed under gentle vacuum followed by incubation under an argon atmosphere. The results demonstrated that NO does, in fact, bind reversibly to both the Fe−TfdA and Fe−TfdA·(α-KG) complexes (most NO exchanged within 10 min). Under these same conditions, however, NO was *not* released from the ternary Fe−TfdA·(α-KG)·(2,4-D) complex. This increased affinity for NO, and by analogy O_2, in the presence of substrate(s) was observed previously in other O_2-activating non-heme Fe(II) enzymes (*59*, *60*) and presumably protects the enzyme from self-inactivation since the formation of reactive Fe−O_2 species is controlled.

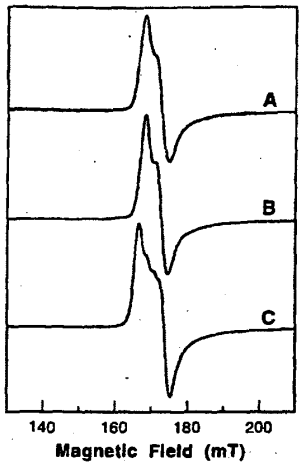


FIGURE 10: EPR spectra of Fe−TfdA-nitrosyl complexes. Only the expanded g = 4 region is displayed since the g = 2 region is relatively uninformative and there was frequent overlap with the signal of free NO. (A) Fe−TfdA·(NO) (g_x = 4.09, g_y = 3.98, g_z = 2.00, g_x to g_y peak-to-peak width of 6.4 mT); (B) Fe−TfdA·(α-KG)·(NO) (g_x = 4.09, g_y = 3.98, g_z = 2.00, g_x to g_y peak-to-peak width of 6.4 mT); (C) Fe−TfdA·(α-KG)·(2,4-D)·(NO) (g_x = 4.12, g_y = 3.96, g_z = 2.00, g_x to g_y peak-to-peak width of 8.9 mT).

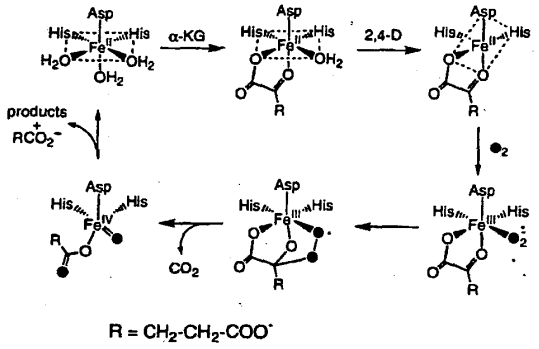
DISCUSSION

TfdA exhibits many features associated with the emerging class of mononuclear non-heme iron enzymes that possess a 2-His-1-carboxylate facial triad (*19*). While these enzymes activate dioxygen to catalyze a diverse array of oxidative transformations, they share a common structural motif in which two histidines and one carboxylate occupy a trigonal face of the metal coordination sphere (Figure 1). This facial triad is proposed to anchor the iron(II) center in the active site, leaving the remaining coordination sites available for binding exogenous ligands such as cofactor, substrate, and/or O_2 (*19*). Such an arrangement allows the enzyme to juxtapose reactants to facilitate catalysis. In addition, it has been suggested that ligating anionic exogenous ligands may prime the iron center for binding and activating O_2, presumably by altering the redox potential of the metal center (*19*). Scheme 4 shows a proposed mechanistic scheme for TfdA based on our results as well as information from other mechanistically related enzymes and model systems (*2*, *8*,

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Scheme 4: Proposed Reaction Scheme for TfdA Based on Current TfdA Studies and Published Work with Related Enzymes



9, *20*–*25*, *31*). Evidence supporting this scheme is discussed in the following sections.

2-His-1-Carboxylate Facial Triad. Our EPR and ESEEM studies of Cu–TfdA and the binary Cu–TfdA·(α-KG) complex demonstrate that two histidine ligands coordinate the metal center in the equatorial plane. In the EPR spectra, both the lowest $g_{\parallel}$ feature and the $g_{\perp}$ signal exhibit 5-fold splitting, indicative of superhyperfine splitting from two nitrogen ligands. These two nitrogen ligands were identified on the basis of characteristic ESEEM features as arising from the distal nitrogens of two or more coordinated histidine ligands (*32*). The present results are consistent with extended X-ray absorption fine structure (EXAFS) spectroscopic data (*31*) that identified two histidine ligands to both the Cu–TfdA and the Fe–TfdA metallocenters.

Sequence comparisons show that IPNS and a subset of the α-keto acid-dependent enzymes share a common His-X-Asp-$X_{(53-57)}$-His motif (*30*). On the basis of numerous spectroscopic and biochemical studies, it was proposed that this motif provides the endogenous ligands to the metal center (*19*), a notion later confirmed crystallographically for IPNS (*18*) and DAOCS (*8*, *9*). TfdA also contains a His-X-Asp-X_{51}-His sequence (His113, Asp115, and His167) (*29*), although it does *not* possess obvious overall sequence similarity to IPNS or DAOCS. Furthermore, mutagenesis studies on TfdA have demonstrated that the residues associated with this motif are essential for enzyme activity.[3] Together with the EPR, ESEEM, and EXAFS data, these results provide very strong evidence that TfdA utilizes the 2-His-1-carboxylate facial triad as the endogenous metal ligand set.

Exogenous Ligands. A key aspect of the 2-His-1-carboxylate facial triad is that this endogenous ligand set maintains three cis-oriented sites which are available for the binding of exogenous ligands and are proposed to play a critical role in the catalytic mechanisms of these enzymes (*19*). By analogy to DAOCS (*9*), these sites are presumably occupied by solvent molecules in the resting state of TfdA. Indeed, ESEEM spectroscopic results of Cu–TfdA in H_2O and D_2O are consistent with the presence of one axial and two equatorial water molecules. Significantly, the EPR spectrum of Cu–TfdA obtained in the presence of imidazole buffer shows evidence for imidazole binding to the metal center, presumably displacing one or two solvent molecules from the principal tetragonal plane.

The addition of the cosubstrate α-KG to Fe(II)-reconstituted TfdA results in the displacement of two coordinating solvent ligands as shown by the development of MLCT transitions that are diagnostic of an α-keto acid moiety chelated to an Fe(II) center (*20*–*22*, *25*). Indeed, the crystal structure of the Fe-DAOCS·(α-KG) complex reveals just such a chelated α-KG (*9*). A chelated α-KG was also recently deduced for the Fe(II)-reconstituted α-keto acid-dependent enzymes CS2 (*23*) and TauD (*64*).[4] Thus, the chelate binding mode of α-KG appears to be general to all α-keto acid-dependent enzymes. The proposed binding orientation for α-KG shown in Scheme 4 corresponds to that observed in the crystal structure of DAOCS.

The optical spectrum of the ternary Fe–TfdA·(α-KG)·(2,4-D) complex retains MLCT transitions characteristic of an α-keto acid chelated to an Fe(II) ion, indicating that α-KG remains chelated to the iron center in the presence of the substrate 2,4-D. The addition of substrate does, however, alter the coordination environment of the active site as evidenced by the blue-shift in the MLCT transitions and the sharpening of the optical spectrum. Analogous perturbations to the MLCT transitions also occur in the spectra of both Fe–CS2·(α-KG) (*24*) and Fe–TauD·(α-KG) (*64*) upon addition of the appropriate substrate, suggesting that this perturbation may be a feature common to all α-keto acid-dependent enzymes. We interpret the sharpening of the optical features as an indication that the iron is in a more homogeneous environment in the presence of both α-KG and 2,4-D, and that the Fe(II) ion is now "locked" in position and primed for catalysis.

In contrast to the Fe(II)-reconstituted enzyme, addition of α-KG to Cu(II)-substituted TfdA results in the displacement of only a single equatorial water molecule according to ESEEM analysis, suggesting that α-KG binds in a monodentate fashion to Cu–TfdA. This difference in α-keto acid-binding mode between Cu(II) and Fe(II) adducts, which has also been observed in model complexes (*65*), is not surprising given the preference of Cu(II) to maintain a tetragonal coordination environment. Upon the addition of 2,4-D to the Cu–TfdA·(α-KG) complex, a second equatorial water molecule is lost. It remains unclear whether the water is displaced by the α-keto group or by direct ligation of the substrate, but we favor the former interpretation which would allow the primary substrate to bind identically in the Cu- and Fe-enzymes.

Creation of an O_2-Binding Site. As illustrated in Scheme 4, one feature associated with priming the Fe(II) site for reaction with O_2 may entail the loss of a ligand to form a coordinately unsaturated metal center. Our EPR studies of various Cu–TfdA and nitrosyl-Fe–TfdA complexes indicate that a significant change occurs in the metal environment upon formation of the Cu/(Fe–NO)–TfdA·(α-KG)·(2,4-D) complex, with both spectra exhibiting more rhombicity. In addition, for Cu–TfdA·(α-KG)·(2,4-D), we observe a low field shift of $g_{\parallel}$ to 2.37, a decrease of $A_{\parallel}$ to 12.0 mK, and a

[3] Hogan, D. A., Smith, S. R., Saari, E. A., McCracken, J., and Hausinger, R. P., manuscript in preparation.

[4] TauD catalyzes the conversion of taurine to aminoacetaldehyde and sulfite [Eichhorn et al. (1997) *J. Biol. Chem. 272*, 23031–23036].

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sharpening of the $g_{||}$ signals by a factor of 2 due to the loss of superhyperfine splitting from one nitrogen ligand. The first two spectral changes indicate a Cu(II) ion with more oxyanionic ligands in the principal tetragonal plane (*44*), while the third indicates the displacement of one His ligand from this plane (shown by the shift in position of the dotted lines in Scheme 4). Two options can be considered for this His displacement: complete loss of a His ligand from the coordination sphere or a reorientation of the principal g-tensor axis.

In support of a model in which one His ligand is lost upon addition of the substrate 2,4-D, EXAFS spectra of the ternary Cu−TfdA·(α-KG)·(2,4-D) and binary Fe−TfdA·(2,4-D) complexes show decreases in the intensity of outer-sphere features due to imidazole coordination (*31*). It should be stressed, however, that precise numbers of ligands are difficult to assign by this method, especially involving second-shell atoms, and that EXAFS spectra for the ternary Fe−TfdA·(α-KG)·(2,4-D) complex were not reported (*31*). The EXAFS data suggest that 2,4-D binding leads to His displacement but do not define whether the ligand changes arise from protein conformational changes associated with substrate binding or whether the carboxylate of 2,4-D replaces the endogenous ligands. In the latter case, this proposed displacement would be specific only for substrates with carboxylate groups and not apply to either prolyl hydroxylase or lysyl hydroxylase, two α-keto acid-dependent enzymes involved in collagen formation (*1*). A further argument against this model is the crystal structure reported for DAOCS. If the active site of DAOCS is representative of that in TfdA, the displacement of a histidine in the facial triad would appear to require substantial side-chain rearrangements (*8*, *9*).

We currently favor an alternative interpretation in which the endogenous 2-His-1-carboxylate facial triad is maintained upon 2,4-D binding. According to this alternative proposal, α-KG binds iron in a chelate fashion while 2,4-D binds in a pocket near the iron center and induces the loss of a solvent molecule from the metal coordination sphere, resulting in a 5-coordinate iron(II) metallocenter. This interpretation is in agreement with the perturbation observed at the iron(II) center upon addition of 2,4-D to Fe−TfdA·(α-KG), as well as the crystal structure (*9*) and modeled substrate binding pocket (*8*) of DAOCS. Further support of this model is provided by detailed spectroscopic studies of CS2 (*23*, *24*) in which Solomon and co-workers demonstrated a shift from a 6- to 5-coordinate Fe(II) center upon substrate binding. In the case of Cu−TfdA, we propose that the interactions with α-KG and 2,4-D cause a reorientation of the principal tetragonal plane in Cu−TfdA, resulting in a shift of one of the histidines from the tetragonal plane to an axial position (Scheme 4). This reorientation increases the number of oxygen ligands in this plane and decreases the multiplicity of the superhyperfine splitting, consistent with the Cu−TfdA EPR data.

As Scheme 4 illustrates, having formed the ternary Fe−TfdA·(α-KG)·(2,4-D) complex, the next step is to bind O_2. Evidence that the addition of substrate to the binary complex primes the iron to bind and activate O_2 can be derived from studies with the O_2 mimic NO. Nitric oxide binding is reversible unless both α-KG and 2,4-D are present, suggesting that the ternary complex has a much higher affinity than the binary or free enzyme for NO and, by analogy, O_2. An energetic coupling of NO/O_2 and substrate binding has been observed previously in other non-heme iron enzymes that activate O_2, including three different extradiol dioxygenases (*59*, *60*) and IPNS (*61*). The increased affinity for NO/O_2 only in the presence of a properly "primed" active site presumably serves to protect the enzyme since the potent oxidizing species will only be generated when the catalytic cycle can be completed (*19*). This hypothesis is consistent with the ordered binding mechanism established for the α-keto acid-dependent enzyme thymine 7-hydroxylase (*4*), where O_2 binds after both α-KG and the primary substrate and the fact that the oxidative decarboxylation of α-KG cannot be uncoupled from 2,4-D oxidation in TfdA (*27*). In addition, stopped-flow studies with TauD demonstrate an enhanced rate of reaction with O_2 for the ternary complex relative to Fe−TauD·(α-KG), suggesting a similar priming of the active site upon the addition of both α-KG and the primary substrate (*64*).

Mechanistic Considerations. Understanding the coordination environment of the iron(II) center in TfdA ultimately leads to the question of how α-keto acid-dependent enzymes activate O_2. Cytochrome P-450, the prototypical heme oxygenase, utilizes an electron from NADH and one from the porphyrin ring itself to generate compound I, the putative high valent iron−oxo species involved in oxidation (*66*). In the non-heme methane monooxygenase, the dinuclear iron activates O_2 to form intermediate Q, proposed to be a diiron-(IV)-oxo species (*67*). Mononuclear, non-heme iron enzymes, however, must utilize the exogenous ligands themselves to supply the additional electrons necessary to activate and reduce O_2 (*19*). Thus, the extra electrons are furnished by the cosubstrate α-KG in α-keto acid-dependent enzymes. Still left unanswered, however, is how these enzymes overcome the energy required to break the O−O bond. In heme enzymes, it is generally accepted that O−O bond cleavage is induced by the "push−pull" mechanism, with the respective involvement of the proximal and distal residues (*66*). No such mechanism is available in the α-keto acid-dependent oxygenases. Analysis of the reactivity of model complexes suggests that the oxidative decarboxylation itself may provide the necessary driving force; that is, the loss of CO_2 may be coupled to O−O bond cleavage as depicted in Scheme 4 and, therefore, provides the energy required to overcome this key activation barrier (*20*). Thus, the α-keto acid cosubstrate would have a unique role, providing both electrons and an energetic boost.

In conclusion, the spectroscopic results reported herein have greatly expanded our knowledge of the iron coordination environment in α-keto acid-dependent enzymes. Our current working model for TfdA, shown in Scheme 4, builds on a hypothetical mechanism first proposed for prolyl hydroxylase by Hanauske-Abel in 1982 (*26*). The scheme consists of an iron coordinated to the active site via the widely used 2-His-1-carboxylate facial triad with the remaining sites filled with solvent molecules and available for the binding of exogenous ligands. α-KG chelates to the iron via the α-keto oxygen and a carboxylate oxygen forming a stable five-membered ring. This binding mode is maintained even in the presence of the primary substrate 2,4-D, the binding of which induces the loss of a solvent ligand to generate a 5-coordinate iron(II) center. Upon the binding of O_2 at the

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vacant site, α-KG is oxidatively decarboxylated, releasing both the electrons and the driving force necessary to form the putative high valent iron−oxo intermediate. Thus, the unique coordination environment of α-keto acid-dependent enzymes affords an oxygen activation mechanism, which is distinct from those utilized by oxygenases which require a porphyrin ring or a second metal ion.

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Biochemistry

Modification of the coding sequence enhances plant expression of insect control protein genes

(*Bacillus thuringiensis*/insect-resistant plants/synthetic genes)

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ABSTRACT **Increased expression of the insect control protein genes of *Bacillus thuringiensis* in plants has been critical to the development of genetically improved plants with agronomically acceptable levels of insect resistance. The expression of the *cryIA(b)* gene was compared to partially modified (3% nucleotide difference) and to fully modified (21% nucleotide difference) *cryIA(b)* and *cryIA(c)* genes in tobacco and tomato. The modified genes increased the frequency of plants that produced the proteins at quantities sufficient to control insects and dramatically increased the levels of these proteins. Among the most highly expressing transformed plants for each gene, the plants with the partially modified *cryIA(b)* gene had a 10-fold higher level of insect control protein and plants with the fully modified *cryIA(b)* had a 100-fold higher level of CryIA(b) protein compared with the wild-type gene. Similar results were obtained with the fully modified *cryIA(c)* gene in plants. Specific sequences of the partially modified *cryIA(b)* gene were analyzed for their ability to affect *cryIA(b)* gene expression in tobacco. The DNA sequence of a single region was identified as important to the improvement of plant expression of the *cryIA(b)* gene. The increased levels of *cryIA(b)* mRNA were not directly proportional to the increased levels of CryIA(b) protein in plants transformed with the modified *cryIA(b)* genes, indicating that the nucleotide sequence of these genes had an effect in improving their translational efficiency in plants.**

Insect control proteins from a prokaryotic source, *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.*; ref. 1) are specific for lepidopteran insects and exhibit no activity against humans, other vertebrates, and beneficial insects (2). These properties have made the genes of these insect-specific proteins attractive candidates for genetic modification of crops for protection against lepidopteran pests. Genes encoding lepidopteran-specific insect control proteins have been cloned and sequenced. Truncated genes, which produce insecticidally active protein, have been expressed in tomato (3), tobacco (4), and cotton (5). Field tests of these plants revealed that higher levels of insect control protein in the plant tissue would be required to obtain commercially useful plants (6).

The insect control proteins are highly expressed in their natural host, *B. thuringiensis*. Up to 50% of the total protein in sporulated cultures of *B.t.k.* are the insect control proteins deposited as crystals within the cell. Insect control protein genes are expressed well in *Escherichia coli* (7) or *Pseudomonas* (8). Poor expression in plants is a well-reported characteristic of the *B.t.k.* insect control proteins. Truncating the gene, keeping essentially the N-terminal half of the protein intact, results in improved expression of the gene in plants to barely detectable levels (3, 4). The use of different promoters, fusion proteins, and leader sequences has not significantly increased insect control protein gene expression (4, 9).

We hypothesized that a gene with a sequence adapted for a Gram-positive prokaryote may not be the appropriate coding sequence for efficient plant expression. Examination of the insect control protein gene coding sequence indicated that it differs significantly from plant genes in G+C content. Multiple sequences motifs that are not common in the coding region of plant genes were found to be common in the wild-type (WT) *cryIA(b)* sequence. These included localized regions of A+T richness resembling plant introns (10), potential plant polyadenylylation signal sequences (11), ATTTA sequences, which have been shown to destabilize mRNA in other systems (12), and codons rarely used in plants (13).‡

Two approaches were initiated to increase the level of CryIA(b) and CryIA(c) insect control proteins in genetically modified plants. First, DNA sequences predicted to inhibit efficient plant gene expression at both the translational and mRNA level were selectively removed throughout the coding sequence by site-directed mutagenesis to partially modify the gene (PM gene) without changing the amino acid sequence. Wholesale changes in the DNA characterized the second approach, which required the use of a fully modified (FM) synthetic gene. The FM genes encode proteins nearly identical in amino acid sequence to the WT gene. These constructs were a more rigorous application of the principles used to generate the PM gene, taking into account additional factors such as codon usage in plants, potential secondary structure of mRNA, and potential regulatory sequences. Insect control protein levels in plants increased as a consequence of modification of the DNA sequence, up to 100-fold over levels seen with the wild-type gene in plants. The increased gene expression appears to be the result of increased translational efficiency. The higher level of insect control protein in these plants was directly correlated to increased insecticidal activity. The demonstration of the utility of these genes to provide protection from insects has far-reaching implications for the future of insect-resistant plants and for the application of these gene modification principles to the design of heterologous genes for high-level expression in plants.

MATERIALS AND METHODS

Modifications of the Coding Sequence of Insect Control Genes. The coding sequence of the WT gene in pMON9921

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Abbreviations: WT, wild-type; PM, partially modified; FM, fully modified; THW, tobacco hornworm; *B.t.k.*, *Bacillus thuringiensis* var. *kurstaki*.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M60856).

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was identical in DNA sequence and similar to the truncated version of *cryIA(b)* (3). Modifications in the DNA did not alter the amino acid sequence of the CryIA(b) protein. Partial modification of the WT sequence was by site-directed mutagenesis (14) with 9 oligonucleotides, labeled A–I as they appear in the sequence. The nucleotides that differ from the WT sequence are shown in Fig. 1. Regions of the coding sequence that were targeted for modification were identified by scanning the DNA sequence for 4 or more consecutive adenine (A) or thymine (T) nucleotides. These regions were analyzed for the presence of potential polyadenylylation signal sequences (11). If there was more than 1 signal sequence within 10 nucleotides, the sequence was altered. If any region of 15–30 nucleotides was >80% A+T, it was altered if there were 2 or more ATTTA sequences or if there was a major potential plant polyadenylylation signal sequence (AATAAA or AATAAT). Nucleotides used to modify the DNA sequence increased the overall G+C content, increased plant preferred codons (13) without changing the amino acid sequence, and did not generate consecutive A+T or G+C strings (>6).

The changes of the FM gene [FM *cryIA(b)*] required complete synthesis (British Biotechnology, Oxford, U.K.). The DNA sequence was designed to maintain the amino acid sequence and avoid ATTTA sequences and potential polyadenylylation signal sequences. Plant preferred codons were used. Consecutive strings of A+T or G+C nucleotides of 5 or more were avoided. The FM *cryIA(b)* gene is compared to the WT in Fig. 1. The FM *cryIA(c)* gene (5) is a synthetic truncated version of the *cryIA(c)* gene and encodes amino acids 1–615. A second FM *cryIA(c)* gene, described in Table 2, encodes amino acids 29–615.

Plant Expression Vectors. The genes for insect control were delivered as cointegrates of a disarmed nopaline plasmid (15). These intermediate vectors all contain the insect control protein genes [WT, PM, FM, or FM *cryIA(c)*] driven by the cauliflower mosaic virus 35S promoter with a duplicated enhancer region (16).

Plant Transformation and Regeneration. *Agrobacterium tumefaciens* was used to obtain transgenic tobacco (17) and tomato (18) plants.

Immunological Analysis. Western blot analysis was done as described (5). Antibodies prepared in rabbits to truncated, denatured insect control protein [trypsin-treated CryIA(c) protein] isolated from a SDS/polyacrylamide gel were used. The blots were sensitive to 1–2 ng per 50 μg of total plant protein. ELISA analysis of transgenic plant material for CryIA(b) protein utilized a direct double antibody sandwich with rabbit polyclonal antibody raised to the *cryIA(b)* gene product as expressed in *E. coli*, purified as described (19). The ELISA was sensitive to 0.5 ng per 50 μg of total protein.

mRNA Analysis. S1 nuclease protection assays (20) were used to quantitate the levels of *cryIA(b)*-specific mRNA. A ^{32}P kinase-labeled 45-mer synthetic oligonucleotide with 30 nucleotides of homology to the 5′ untranslated sequence common to all of the insect control protein gene plant mRNAs was hybridized with 40 μg of total RNA. An internal control of a labeled 55-mer with 40 nucleotides of homology to the coding sequence of the endogenous tomato 3-*enol*-pyruvoylshikimate-5-phosphate synthase (EPSPS) mRNA (21) was included in each reaction mixture. Each lane was scanned with a laser densitometer for quantitation and was normalized with respect to the levels of endogenous EPSPS mRNA. Several exposures were scanned to account for film saturation.

Insect Bioassay. Five neonate larvae of tobacco hornworm (THW) were placed on a leaf of a plant that had been selected in tissue culture for kanamycin resistance. Insect control was defined as plant tissue that exhibited 100% mortality to THW and sustained no visible damage after 3 days.

RESULTS

Partial modification of the WT coding sequence focused on potential plant polyadenylylation signal sequences and ATTTA sequences in regions of high local A+T content. The resulting gene, referred to as the PM gene, contained a subset of the changes made in the FM gene (Table 1 and Fig. 1). The design of the FM gene eliminated ATTTA sequences, almost all potential plant polyadenylylation sequences, greatly increased the G+C nucleotide content of the gene, and minimized the use of rare plant codons. The PM gene encodes amino acids 29–607 of the CryIA(b) protein with an additional two amino acids, methionine and alanine, at the N terminus. The FM gene encodes amino acids 1–615. Both genes have been expressed in *E. coli* and the proteins show equivalent activity against lepidopteran insects (data not shown).

Insect Control Protein Levels in Genetically Modified Plants. The plants expressing the WT, PM, or FM genes were identified from the transformed kanamycin-selected plants by a primary screen bioassay against THW (Table 2). No observable damage to the leaf by THW indicated that ingestion of small amounts of leaf tissue by THW was sufficient to kill the insects. The frequency of transgenic plants that exhibited insect control was higher in the PM and FM transformed plants than in the plants with the WT *cryIA(b)* gene (Table 2). The modified genes (PM and FM) were routinely expressed above the biological threshold set for insect control in tomato and tobacco. This occurrence was less frequently observed with the WT gene, especially in tobacco.

Immunological analysis of plants expressing either the WT, the PM, or the FM *B.t.k.* insect control protein genes demonstrated that modification of the *B.t.k.* gene resulted in increased insect control protein levels in both tobacco and tomato. The distribution of these plants by the levels of insect control protein found in leaf tissue revealed that the PM and FM genes had a greater percentage of plants with higher levels of detectable protein compared to transgenic plants with the WT gene (Fig. 2). Over 200 transgenic tomato plants with the WT *cryIA(b)* gene were screened; 53 showed insect control and in only 3 plants were the levels of protein detectable by Western blot analysis. Insect control protein was barely detectable in 2 plants, near the limit of detection in this assay (≈1 ng per 50 μg of total protein).

Tomato plants expressing the PM gene showed an increase in the percentage of insecticidal plants (Table 2) and in the levels of insect control protein detected (Fig. 2*A*). The majority of the plants transformed with the PM gene fall into the 1- to 10-ng level and a small percentage (2 of 25 plants) of the plants are in the lower end of the 10- to 30-ng range (0.02%).

Tomato plants that expressed the FM genes contained a similar percentage of insecticidal plants compared to plants with the PM gene (Table 2). A substantial number of plants (10 of 29; 34%) were found to contain high insect control protein levels (Fig. 2*A*). Slightly higher overall levels of insect control protein were seen with tomato plants transgenic with

Table 1. Summary of changes made in the PM and FM *cryIA(b)* genes compared to the WT *cryIA(b)* gene

	WT	PM	FM
No. of bases different from WT	—	62 of 1743	390 of 1845
No. of codons different from WT	—	55 of 579 (9.5%)	356 of 615 (60%)
G+C content	37%	41%	49%
No. of potential PPSS	18	7	1
No. of ATTTA sequences	13	7	0

PPSS, plant polyadenylylation signal sequences.

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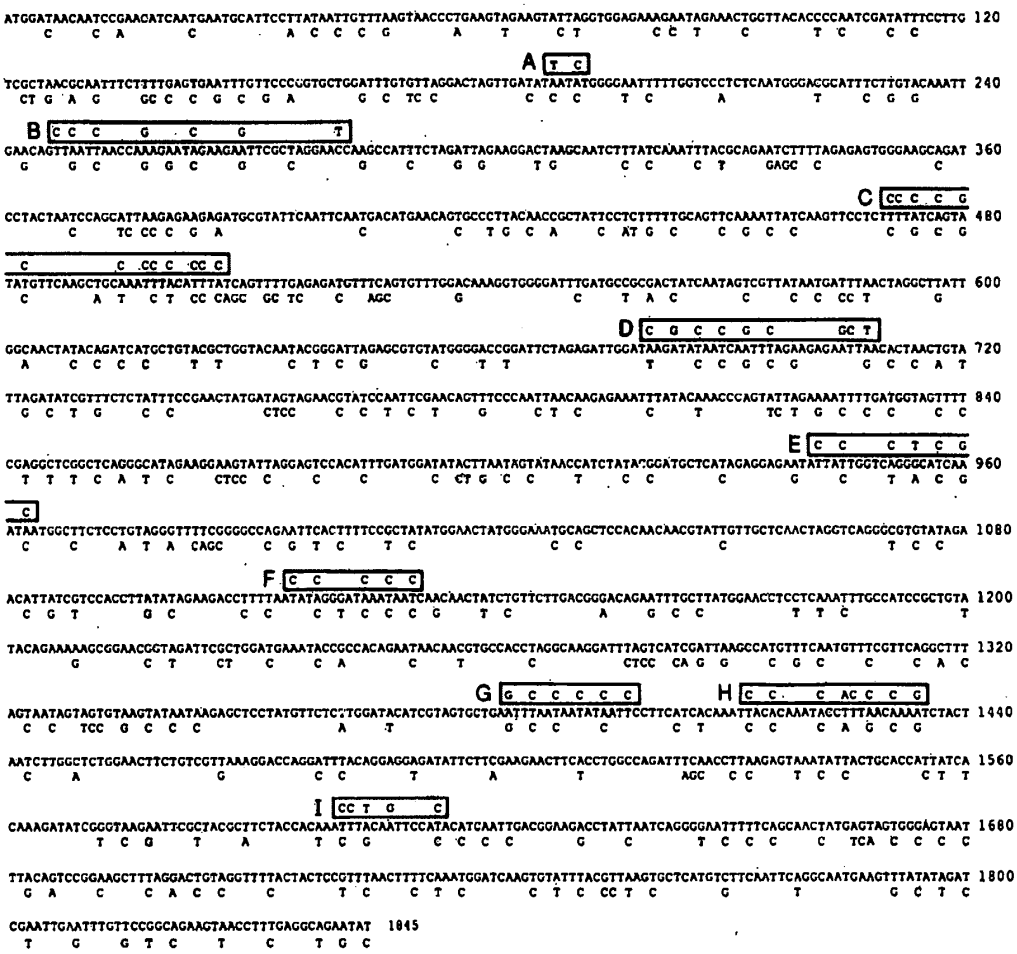


FIG. 1. DNA sequence of the *cryIA(b)* gene on the numbered line with the modifications found in the PM gene on the line above and the FM gene on the line below. The differences between the WT and PM genes are within the labeled boxed areas (A–I).

pMON5383, a truncated *cryIA(c)* FM gene with substantial homology to the *cryIA(b)* FM gene depicted in Fig. 1. Among the 52 FM *cryIA(c)* transgenic tomato plants that were immunologically analyzed, 10 (20%) were at or above the 30- to 100-ng range with several plants approaching 100 ng per 50 μg of total protein. Among the most highly expressing transgenic tomato plants for each insect control protein gene, the FM genes appeared to be expressed 5- to 10-fold higher than the PM gene and up to 100-fold higher than the WT gene.

Table 2. Comparison of the fraction of plants exhibiting insect control with the WT, PM, and FM genes

Gene	Plants exhibiting insect control/ total kanamycin-selected plants	
	Tobacco	Tomato
WT *cryIA(b)*	3/54 (6%)	53/204 (26%)
PM *cryIA(b)*	25/42 (60%)	40/63 (63%)
FM *cryIA(b)*	12/22 (55%)	32/55 (58%)
FM *cryIA(c)*	—	52/97 (54%)
FM *cryIA(c)**	48/95 (51%)	—

*The coding region of the N-terminal amino acids of this FM *cryIA(c)* gene has been deleted as in the WT and PM *cryIA(b)* genes.

In tobacco plants, the WT gene was more poorly expressed than in tomato, below the detection limit of the Western blot analysis in the 3 plants showing insect control against THW. Analysis of transgenic tobacco plants (Fig. 2*B*) with the PM and FM genes revealed results similar to those found in tomato. There was an increase in the frequency of plants with insect control (Table 2) and the levels of insect control protein in plants with the PM gene compared to the WT. As in tomatoes, several tobacco plants (2 of 25; 8%) with the PM gene contained 10–30 ng of insect control protein per 50 μg of total plant protein.

Transgenic tobacco plants with the FM gene demonstrated improved expression of the *B.t.k.* protein over the PM gene plants. Several tobacco plants with the FM genes contained detectable levels of insect control protein >50 ng per 50 μg of total protein (>0.1%). Fig. 2*B* shows a high percentage of tobacco plants with the FM *cryIA(b)* gene in the 30- to 100-ng range but only 13 plants with this construct were analyzed. Extensive analysis was done with pMON5390, a FM trun-

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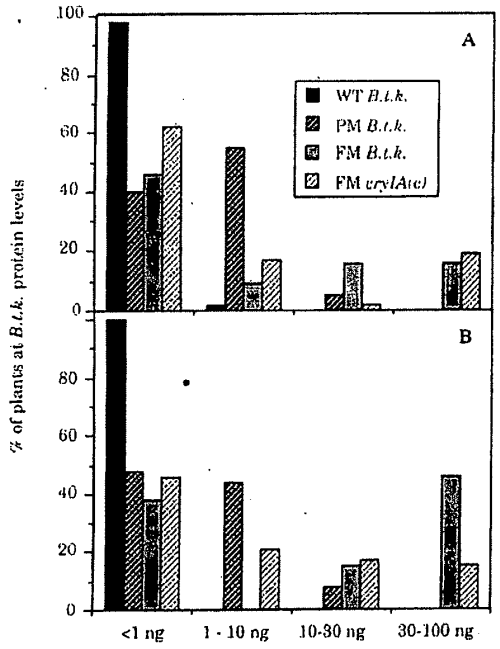


FIG. 2. Distribution of tomato (*A*) and tobacco (*B*) plants that exhibited insect control. The numbers of tomato plants that exhibited insect control used to calculate the percentage of *B.t.k.* protein levels are as follows: WT gene, 53; PM gene, 63; FM gene, 55; FM *cryIA(c)*, 97. The numbers of tobacco plants are as follows: WT gene, 3; PM gene, 42; FM gene, 22; FM *cryIA(c)*, 95. Levels of insect control protein are per 50 μg of total plant protein.

cated *cryIA(c)* gene similar to the *cryIA(c)* gene found in pMON5383 except that the coding sequence for the first 28 amino acids had been deleted as in the WT and PM genes. A high percentage of the plants (50%) that exhibited insect control contained protein at detectable levels. As with the FM gene in tomato, one-third (31%) of the plants were in the 10- to 100-ng range of insect control protein per 50 μg of total protein.

The higher frequency of transgenic plants with the PM and FM genes exhibiting insect control compared to the plants with the WT gene was a good indication that the modifications to the *B.t.k.* coding sequence had improved gene expression. Screening of a large number of plants with the WT gene failed to yield any that produced >1 ng of insect control protein per 50 μg of total protein, while >10% of the plants with the FM genes produced 30–100 ng of insect control protein in both tomato and tobacco. Because of the large numbers of plants analyzed, the overall similarity of the genetic constructs and the large variance in both average and peak expression obtained with the WT, PM, and FM genes, we believe that the data reflected inherent gene expression differences. Gene copy number did not appear to differ for the different coding sequences. The most highly expressing tomato plants containing either the WT, PM, or FM gene were analyzed genetically and the insect control protein gene segregated as a single dominant locus. Highly expressing tobacco and tomato plants with the FM *cryIA(b)* and FM *cryIA(c)* genes were identified that contained insect control protein at levels of 100–150 ng (0.2–0.3%).

mRNA Analysis. The levels of *cryIA(b)*-specific mRNA in plants did increase in plants with the PM gene and the FM gene compared to plants with the WT gene (data not shown). Quantitative S1 nuclease mRNA analysis revealed that selected tomato plants with the PM gene had a 10-fold increase in insect control protein levels and an average 2.5-fold increase in *cryIA(b)* mRNA over tomato plants with the WT gene. Tomato plants with the FM *cryIA(b)*, which exhibited a 50-fold increase in insect control protein levels over tomato plants with the WT gene, had a 5-fold increase of *cryIA(b)* mRNA levels. For two selected plants, levels of mRNA from the plant with the PM gene differed by <20% from that in a plant with the FM gene, yet the plant with the FM gene contained a 4-fold higher level of insect control protein. Northern blot analysis of the best plants with the WT gene and plants with the PM and FM *cryIA(b)* genes indicated the *cryIA(b)* mRNA was of the expected size.

Identification of Important Regions for *B.t.k.* Gene Expression in the PM Gene. The FM gene contains extensive differences compared to the WT gene (Table 1). The PM gene differs from the WT gene by 3% of the nucleotides (Fig. 1), yet these changes account for at least a 10-fold increase in insect control protein levels in tobacco. Plasmids containing a fraction of the total changes (Table 3) were used to obtain transformed, kanamycin-selected tobacco plants that were screened by insect bioassay. *B.t.k.* protein levels comparable to the PM gene (pMON5370) were obtained when the changes in the 5′ half of the coding sequence were present (pMON10707). The gene with the changes in the 3′ half alone (pMON10706) did not differ from the WT gene; there was no immunologically detectable insect control protein. Changes with oligonucleotide B (pMON10537) appeared to be the most significant section to elicit the increased insect control protein levels.

The changes made by oligonucleotide B were not the sole effector of increased levels of insect control protein in plants. A construct that contained a fully modified 5′ one-third of the gene, including oligonucleotide B (pMON5378), resulted in plants with levels of insect control protein comparable to plants with the PM gene. By comparison, replacing the first one-third of the FM gene with the homologous WT section (pMON10538) resulted in lower levels of insect control protein than previously observed for the FM gene. The expression of the hybrid gene of pMON10538 indicated that it is possible to achieve increased *B.t.k.* expression compared to the WT gene without altering the region of oligonucleotide B. These results indicate that a single region is probably not solely involved and that changes throughout the sequence as found in the FM gene were required for the highest levels of expression of the insect control protein gene in plants.

Table 3. Analysis of the contributions of individual regions to increased expression of the *B.t.k.* PM gene in tobacco

Construct	Oligonucleotides	Plants with insect control/plants screened	Protein level, % of total protein
pMON5370	A–I*	23/48 (48%)	0.02–0.03†
pMON10707	A,B,C,D	19/48 (40%)	0.028
pMON10706	E,F,G,H,I	1/53 (2%)	ND‡
pMON10539	A	2/55 (4%)	ND‡
pMON10537	B	17/57 (30%)	0.016
pMON10540	A,B	23/88 (26%)	0.015
pMON10705	C	1/47 (2%)	ND‡
pMON10538	1/3 WT, 2/3 FM	19/62 (30%)	0.016
pMON5378	1/3 FM, 2/3 WT	9/20 (45%)	0.02–0.03†

*The locations of the individual regions (A–I) are depicted in the sequence shown in Fig. 1.
†The levels of CryIA(b) were determined by Western blot analysis.
‡The level of CryIA(b) in these plants was too low to be detected by ELISA or Western blot.

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DISCUSSION

The PM and FM genes have dramatically increased insect control protein levels in plants. This appears to be one of the largest increases in gene expression in any system obtained solely through modification of the coding sequence. The rationale for these designs was based on the elimination of sequences such as potential polyadneylylation signal sequences, ATTTA sequences, and A+T-rich regions. The increased expression was generic across several plant species—tobacco, tomato, cotton (5)—and was obtained with two distinct genes, *cryIA(b)* and *cryIA(c)*.

The PM gene contained a subset of the changes found in the fully modified gene, yet produced large increases in insect control protein levels in tobacco and in tomato (at least 10-fold). The lack of extensive changes in the PM gene (3% of the total nucleotides) and the localization of a region critical to increased expression (oligonucleotide B) argues against the hypothesis that the presence of rare plant codons is primarily responsible for the previously reported low level expression of WT insect control protein genes in plants. Analysis of the region of oligonucleotide B revealed no inverted repeats, palindromic sequences, or other apparent secondary structures that distinguished this region from other regions of the structural gene. This region does contain three potential polyadenylylation signal sequences (two AACCAA and one AATTAA). Northern blot analysis of total RNA did not detect truncated *cryIA(b)* mRNA in plants expressing the WT gene. It is possible that the incomplete functioning of a polyadenylylation signal led to processing without polyadenylylation causing instability. Another possibility is that the region of oligonucleotide B contains an as yet undefined regulatory sequence dependent on the context of its surrounding sequence.

The region of oligonucleotide B is not the sole suppressor of insect control protein gene expression in plants. The fully modified C-terminal two-thirds of the truncated insect control protein gene plus an N-terminal WT one-third containing a sequence unchanged in oligonucleotide B (pMON10538) resulted in levels of insect control protein in plants equivalent to the PM gene or ≈10-fold lower than for the FM *cryIA(b)* gene. One interpretation of these results is that the presence of the WT sequence in the 5′ third of the gene, including the region of oligonucleotide B, depresses the expression of the FM gene ≈10-fold. Alternatively, the aggregate of the changes contained in the 3′ two-thirds of the FM gene could partially compensate for the presence of the WT sequences in the 5′ one-third of the gene. These observations suggest that several factors and regions of the gene are involved in the low levels of insect control protein observed with the WT gene in plants. The highest levels of insect control protein that we have observed in plants were obtained with the FM genes, both *cryIA(b)* and *cryIA(c)*.

The major block to efficient WT *cryIA(b)* gene expression in plants appeared to be translational and not at the level of stable accumulation of *cryIA(b)* mRNA. mRNA levels were increased in plants with the PM and FM genes, but the level of mRNA increase was not directly proportional to the level of protein increase in the best of the PM and FM plants. It is not clear that the increased mRNA levels must be directly proportional to the increased protein expression. Analysis of the mRNA levels of some of the PM and FM plants indicated that comparable amounts of FM mRNA led to the production of more insect control protein than PM mRNA, suggesting that the presence of predominantly plant preferred codons in the FM gene has improved its overall translational efficiency.

The impact of the exceptional levels of expression that the PM and FM genes provide is most apparent in the efficacy these plants exhibit to withstand insect attack. The use of these plants in an integrated pest management system will expand the options of farmers to protect their harvest from insect destruction.

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Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor

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Plant productivity is greatly affected by environmental stresses such as drought, salt loading, and freezing. We reported previously that a *cis*-acting promoter element, the dehydration response element (DRE), plays an important role in regulating gene expression in response to these stresses. The transcription factor DREB1A specifically interacts with the DRE and induces expression of stress tolerance genes. We show here that overexpression of the cDNA encoding DREB1A in transgenic plants activated the expression of many of these stress tolerance genes under normal growing conditions and resulted in improved tolerance to drought, salt loading, and freezing. However, use of the strong constitutive 35S cauliflower mosaic virus (CaMV) promoter to drive expression of *DREB1A* also resulted in severe growth retardation under normal growing conditions. In contrast, expression of *DREB1A* from the stress inducible *rd29A* promoter gave rise to minimal effects on plant growth while providing an even greater tolerance to stress conditions than did expression of the gene from the CaMV promoter.

Keywords: drought tolerance, freezing tolerance, transgenic plants, multigene expression, stress-inducible promoter

Drought, salt loading, and freezing are stresses that cause adverse effects on the growth of plants and the productivity of crops. The physiologic response to these stresses arises out of changes in cellular gene expression. Expression of a number of genes has been demonstrated to be induced by these stresses[1,2]. The products of these genes can be classified into two groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in the stress response[3]. The first group includes proteins that likely function by protecting cells from dehydration, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes[3-5]. The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism[3].

Recently, several different gene transfer approaches have been employed to improve the stress tolerance of plants[6]. The transferred genes included those encoding enzymes required for the biosynthesis of various osmoprotectants[7-9] or those encoding enzymes for modifying membrane lipids[10,11], LEA protein[12], and detoxification enzyme[13]. Each of these experiments involved transfer of a gene encoding a single specific stress-protective protein, expressed from the 35S cauliflower mosaic virus (CaMV) promoter. To investigate the possibility of simultaneously enhancing tolerance toward multiple stresses (i.e., drought, salt loading, and freezing) by gene transfer, we transferred a gene encoding a stress-inducible transcription factor that regulates many genes involved in stress tolerance in *Arabidopsis thaliana*.

Analyses of the expression of dehydration-inducible genes in *Arabidopsis* have indicated that at least four independent signal pathways function in the induction of stress-inducible genes in response to dehydrastion[3]. Two are abscisic acid (ABA)-dependent and two are ABA-independent. One of the two ABA-independent pathways overlaps with that of the cold-response. Several stress-induced genes, such as *rd29A*, are induced through this separate ABA-independent pathway[14-18]. A *cis*-acting element has been identified in the promoter region of the *rd29A* gene and is responsible for dehydration- and cold-induced expression[19]. This sequence (TACCGACAT), termed the dehydration-responsive element (DRE), is essential for the regulation of dehydration-responsive gene expression[19] and is found in the promoter regions of other dehydration- and cold-stress inducible genes[18,20]. The cDNAs encoding the DRE-binding proteins, DREB1A and DREB2A, have been isolated by yeast one-hybrid screening[21]. Both proteins specifically bind and activate transcription of genes containing the DRE sequence in *Arabidopsis*.

Expression of the *DREB1A* cDNA under control of the 35S CaMV promoter in transgenic plants gave rise to strong constitutive expression of the stress-inducible genes, and increased tolerance to freezing, salt, and drought stresses[21]. A cDNA encoding CRT/DRE binding protein (CBP1) was isolated from *Arabidopsis* and when overexpressed also enhanced freezing tolerance[22,23]. However, the overexpression of these genes resulted in severe growth retardation under normal growth conditions. Here, we use the stress-inducible *rd29A* promoter to drive expression of *DREB1A*, with the aim of minimizing the negative effects on plant growth experienced with use of the 35S CaMV promoter. We observed improved stress tolerance of the transgenic plants and much improved growth under nonstressed conditions.

Results

Preparation of transgenic *Arabidopsis*. *Arabidopsis* (Columbia ecotype) plants were transformed with vectors expressing the DREB1A cDNA from either a modified 35S CaMV promoter[24] or the stress-inducible *rd29A* promoter. Eighteen antibiotic-resistant *Arabidopsis*

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transformants carrying the 35S:DREB1A transgene (35S:DREB1A plants) and 43 transformants carrying the *rd29A:DREB1A* transgene (*rd29A*:DREB1A plants) were generated using a vacuum infiltration method[25].

Analyses of the 35S:DREB1A plants. Growth of the 35S:DREB1A transformants was compared with wild-type control plants after 35 (Fig. 1A) and 53 days (Fig. 1B). The transgenic plants demonstrated varying degrees of growth retardation, which we postulated to be due to variation in expression of the *DREB1A* transgene[21]. Three of the 18 transformants showed severe growth inhibition, and were classified as 35S:DREB1Aa plants. Transformants exhibiting lesser degrees of growth retardation were classified as either 35S:DREB1Ab (moderate phenotypic changes) or 35S:DREB1Ac (mild phenotypic changes) plants. The growth retardation affected seed numbers under normal growth (control) conditions. We observed a significant reduction in the number of seeds produced by the 35S:DREB1Aa and 35S:DREB1Ab plants compared with wild-type plants. The number of seeds harvested from the 35S:DREB1Aa plants was less than 1% of the wild-type controls. In contrast, the 35S:DREB1Ac plants produced a number of seeds similar to wild-type plants.

The expression of *DREB1A* and its target genes was analyzed in 35S:DREB1Ab and 35S:DREB1Ac plants and compared with control plants transformed with the pBI121 vector (Fig. 2). In the 35S:DREB1Ab plants, the *kin1, cor6.6/kin2, cor15a, cor47/rd17,* and *erd10* stress-response genes[3,26] were strongly expressed under control conditions, as was the *rd29A* gene. The expression of these genes in the 35S:DREB1Ac plants was only slightly higher than in wild-type plants but was noticeably lower than in 35S:DREB1Ab plants under unstressed conditions. In contrast, we detected no difference in the expression of the stress-inducible *AtP5CS, erd1, rd22,* and *rd29B* genes[14,27–29] between the 35S:DREB1A and wild-type plants. These results indicate that overexpression of the DREB1A protein leads to specific induction of its target stress-response genes, but not of nontarget stress-response control genes under conditions in the 35S:DREB1A plants, and that the level of stress-responsive gene expression correlates with levels of *DREB1A* mRNA.

Analyses of the *rd29A*:DREB1A plants. *rd29A*:DREB1A plants were then examined under growth conditions. The *DREB1A* cDNA was driven by the strong stress-inducible *rd29A* promoter. Nearly all of the *rd29A*:DREB1A plants exhibited slight growth retardation under control conditions (Fig. 3). However, unlike the 35S:DREB1A plants, there were no significant differences in growth retardation between the transgenic *rd29A*:DREB1A plant lines. Moreover, seed numbers for the *rd29A*:DREB1A transformants were similar to wild-type plants grown under control conditions.

The expression pattern of the *DREB1A* and *rd29A* genes was similar between the various *rd29A*:DREB1A transformant plants. Expression of these genes in transgenic plant *rd29A*:DREB1Aa is shown in Figure 4. The *DREB1A* transgene was expressed at levels above the endogenous gene in wild-type plants even under control conditions, yet was strongly induced by dehydration, salt, cold stress, and treatment with ABA in the *rd29A*:DREB1Aa plants. Under control conditions, expression of the *DREB1A* gene in the *rd29A*:DREB1Aa plants was much less than in the 35S:DREB1Aa plants and similar to that in the 35S:DREB1Ac plants (Fig. 4). In contrast, we detected similar strong expression of the *DREB1A* gene in both the *rd29A*:DREB1Aa and 35S:DREB1Aa plants under conditions of stress (dehydration, salt, low temperature, and treatment with ABA). We observed similar patterns of expression of the *rd29A* gene in the *rd29A*:DREB1Aa plants. The *rd29A* gene was expressed at low levels under control conditions and was strongly induced by each of the stress treatments. A similar pattern of expression was observed for other stress-inducible, DREB1A-responsive genes in the *rd29A*:DREB1A transgenic plants (data not shown).

Freezing, drought, and salt stress tolerance of the transgenic plants. The tolerance of the *rd29A*:DREB1A plants to freezing, dehy-

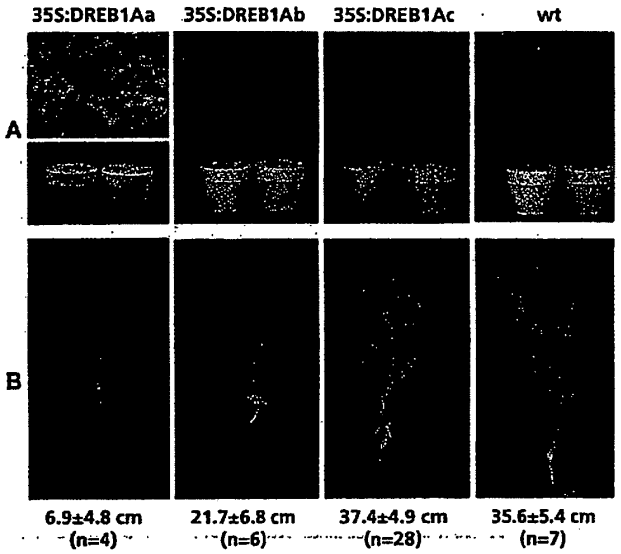


Figure 1. Phenotypes of the 35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac plants in relation to wild-type plants (pBI121). (A) Plants grown for 35 days. (B) Plants grown for 53 days. The average height of each plant line grown for 53 days is indicated under each picture.

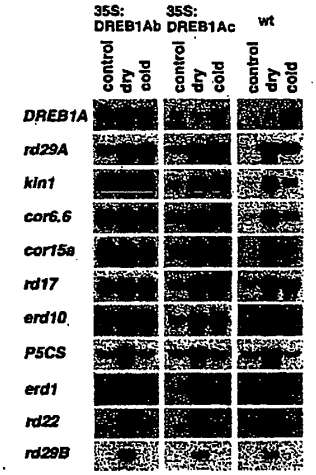


Figure 2. Expression of *DREB1A* target gene mRNAs in 35S:DREB1A transgenic plants and in the wild-type controls. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arbidopsis* plants that had been dehydrated for 5 h (dry), treated at 4°C for 5 h (cold), or left untreated (control). The DNA fragments of the full-length *DREB1A, KIN1, COR6.6, cor15a, RD17, ERD10, AtP5CS, ERD1,* and *RD22* cDNAs and the 3′-terminal specific DNA fragment of *rd29A* and *rd29B* were used as probes.

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dration, and salt stress was compared with that of the 35S:DREB1Ab and 35S:DREB1Ac plants grown in pots or on agar plates at 22°C for 3 weeks (Fig. 5A). When plants grown in pots were exposed to temperatures of -6°C for 2 days, then returned to 22°C and grown for 5 days, less than 10% of the wild-type plants survived, whereas 77.9% and 96.2% of the 35S:DREB1Ab and *rd29A*:DREB1Aa plants survived, respectively (Table 1A). The surviving transgenic plants continued to grow and subsequently flowered. Freezing tolerance of the 35S:DREB1A plants correlated with the level of expression of the stress-inducible DREB1A-responsive genes under control conditions: fewer of the 35S:DREB1Ac plants survived than 35S:DREB1Ab plants (48.8% vs. 77.9% survival, respectively; Table 1A). In contrast, the freezing tolerance of the *rd29A*:DREB1Aa plants was much stronger than that of the 35S:DREB1Ab plants, even though the expression of the stress-inducible DREB1A-responsive genes was weak under control conditions (Fig. 5A and B).

To test whether overexpression of the *DREB1A* gene enhanced tolerance to dehydration, the wild-type and transgenic plants grown in pots were not watered for 2 weeks (Fig. 5A). Nearly all the wild-type plants died within this 2-week period, whereas 69.2% of the 35S:DREB1Ab transgenic plants survived this level of drought stress and continued to grow when watering resumed (Table 1B). The toler-

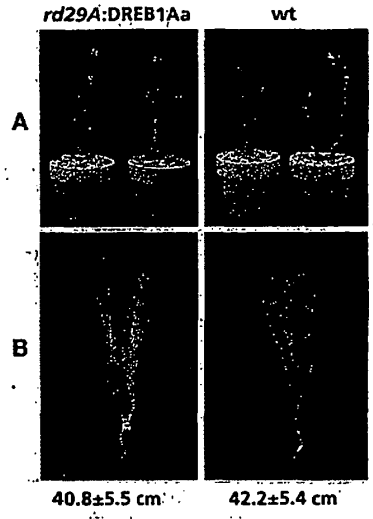


Figure 3. Phenotypes of the *rd29A*:DREB1Aa and wild-type control plants (transformed with the vector pBI121) under control conditions. (A) Plants grown for 30 days. (B) Plants grown for 63 days. The average height of each plant line grown for 63 days is indicated under each picture.

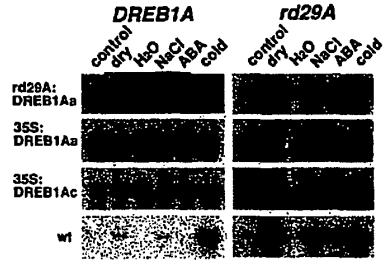
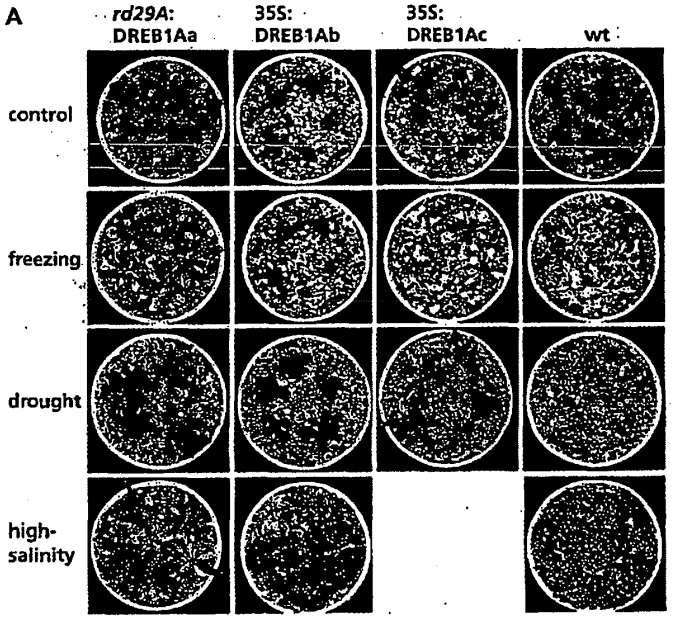


Figure 4. Expression of the *DREB1A* and *rd29A* genes in 35S:DREB1A and *rd29A*:DREB1A transgenic plants. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arbidopsis* plants that had been dehydrated (dry), grown hydroponically in 250 mM NaCl (NaCl), in water (H_2O), or in 100 µM ABA (ABA), or transferred to agar plates at 4°C (cold), or untreated (control). The stress treatments were continued for 5 h.



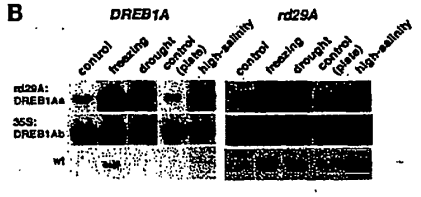


Figure 5. (A) Freezing, drought, and salt stress tolerance of the 35S:DREB1Ab, 35S:DREB1Ac, and *rd29A*:DREB1Aa transgenic plants. The stress treatments were performed as described in the text. Control: 3-week-old plants growing under normal conditions; freezing: plants exposed to a temperature of -6°C for 2 days and returned to 22°C for 5 days; drought: water withheld for 2 weeks; high-salinity: plants soaked in 600 mM NaCl solution for 2 h and transferred to pots under control conditions for 3 weeks. (B) Expression of the *DREB1A* and *rd29A* genes in the 35S:DREB1A and *rd29A*:DREB1A transgenic plants subjected to freezing, drought, and salt stress. Control: 3-week-old plants growing under normal conditions; freezing: plants exposed to a temperature of -6°C for 2 days; drought: water withheld for 5 days; control: plants grown on GM agar plates for 2 weeks; high-salinity: plants that were grown on agar plates for 2 weeks, soaked in 600 mM NaCl solution for 2 h.

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Table 1. Survival rates of transgenic plants.

A. Freezing tolerance

	Survival	Total	%
rd29A:DREB1A	177	184	96.2
35S:DREB1Ab	67	86	77.9*
35S:DREB1Ac	40	82	48.8*
wt	9	95	9.5*

B. Drought tolerance

	Survival	Total	%
rd29A:DREB1A	92	120	76.7
35S:DREB1Ab	45	65	69.2
35S:DREB1Ac	23	58	39.7*
wt	1	55	1.8*

C. High-salinity stress tolerance

	Survival	Total	%
rd29A:DREB1A	125	159	78.6
35S:DREB1Ab	10	34	29.4*
wt	7	39	17.9*

Number of plants surviving exposure to (A) freezing, (B) drought, and (C) high-salinity. Experiments were repeated five times. Five to fifty plants were tested in each experiment. Statistical significance, compared with the value of *rd29A*:DREB1A, was determined by χ^2 test (*$p<0.005$).

ance of the 35S:DREB1A plants to drought stress depended on the level of the expression of DREB1A-responsive genes under control conditions: 39.7%, 69.2%, and 76.7% survival was observed for the 35S:DREB1Ac, 35S:DREB1Ab, and *rd29A*:DREB1A plants, respectively (Table 1B). To examine the tolerance of the transgenic plants to salt stress, plants grown on agar plates were removed from the plates, soaked in 600 mM NaCl solution for 2 h, and then grown in pots for 3 weeks (Fig. 5A). Only 17.9% of the wild-type plants survived this treatment compared with 29.4% of the 35S:DREB1Ab plants (Table 1C). In contrast, the *rd29A*:DREB1A plants were highly tolerant of the salt stress (78.6% survival; Table 1C).

The expression of *DREB1A* and DREB1A-responsive genes was examined in the transgenic plants after freezing, drought, and high-salinity stress treatments (Fig. 5B). The *DREB1A* and *rd29A* genes were weakly expressed even under control conditions in the *rd29A*:DREB1Aa plants. Upon exposure to stress conditions, expression of these genes was elevated to similar levels in *rd29A*:DREB1Aa to that in 35S:DREB1Ab plants (Fig. 5B). These results indicate that strong expression of the DREB1A-responsive genes under stress conditions correlated with tolerance to freezing, drought, and high-salinity stress.

Discussion

Many genes have been demonstrated to respond to drought, high salt levels, and cold stress, and the proteins encoded by these genes are thought to function in protecting cells from these stresses. In the present study, we were able to produce transgenic plants that were highly drought-, salt-, and freezing-tolerant by overexpressing a single gene for a stress-inducible transcription factor, DREB1A. DREB1A binds to the *cis*-acting DRE and regulates the expression of many stress-inducible genes under drought, salt, and cold stress in *Arabidopsis*[21]. The expression of the transferred *DREB1A* gene and its stress-inducible target genes were correlated in the transgenic plants under control conditions. We detected the overexpression of six stress-inducible DREB1A-responsive genes in both 35S:DREB1A and *rd29A*:DREB1A transgenic plants. The genes encoding proteins involved in stress tolerance seem to be overproduced even under control conditions in the 35S:DREB1A transgenic plants, but are rapidly overproduced only in response to stress treatments in the *rd29A*:DREB1A plants. These target genes (*rd29A, kin1, cor6.6/kin2, cor47/rd17, cor15a,* and *erd10*) contain the DRE or related motifs[18–20,22], and are normally induced by dehydration, salt, and cold stresses. In contrast, the *AtP5CS, erd1, rd22,* and *rd29B* genes, which do not contain the DRE or related sequences[27–30] and are not targets of DREB1A, were not overexpressed in *DREB1A* transgenic plants. However, we believe that additional stress-inducible genes may be overexpressed in these transgenic plants under control conditions.

LEA proteins appear during the maturation of embryos and desiccation of seeds, and are also induced by drought, salt, and cold stresses in the vegetative tissues of various plants[31,32]. These proteins are quite hydrophilic and are believed to function by directly protecting plant cells from these stresses. One stress-inducible DREB1A-responsive gene, *rd29A*, encodes a protein similar to the LEA proteins[14]. The *cor47/rd17* and *erd10* genes both encode group 2 LEA proteins[31]. The *kin1* and *cor6.6/kin2* genes encode proteins that are structurally similar to the alanine-rich antifreeze proteins produced by some fish[20]. These similarities in structure and expression suggest that the products of these genes may have similar functions in plants. The *cor15a* gene encodes a protein that is targeted to the stromal compartment of the chloroplasts[33], and enhances the freezing tolerance of *Arabidopsis* leaf protoplasts[34].

Cold acclimation increases the freezing tolerance of plants[2]. Various genes are induced during cold acclimation, such as *rd29A/cor78/lti78, kin1, cor6.6/kin2, cor15a, cor47/rd17,* and *erd10*. The DRE is involved in the induction of these genes in response to low temperatures[1]. Overexpression of *DREB1A* induced expression of these genes under control conditions and increased the freezing tolerance of transgenic plants in a fashion reminiscent of what occurs during cold-acclimation of wild-type plants. Overexpression of CBF1, a DREB1A homolog, both enhanced freezing-stress tolerance and increased the expression of *cor15a, cor6.6,* and *cor47* (ref. 23). These observations demonstrate that the target genes of both the DREB1A and CBF1 encode proteins that function in protecting cells from freezing stress. Overexpression of DREB1A also enhanced drought and salt tolerance in the transgenic plants, demonstrating that the proteins encoded by the target stress-inducible genes also function in protecting cells from drought and salt stress.

The 35S:DREB1A plants exhibited varying degrees of growth retardation under control conditions. The extent of both growth retardation under control conditions and stress tolerance correlated with the level of constitutive expression of the DREB1A transcript and its target genes. These results indicate that stress tolerance of the 35S:DREB1A plants comes at the expense of growth and productivity.

To overcome the problem of growth retardation, we used the stress-inducible *rd29A* promoter to overexpress DREB1A. The *rd29A* promoter is stress-inducible and contains binding sites for the DREB1A protein. This promoter allowed low levels of DREB1A expression during unstressed conditions, yet permitted rapid high-level expression of the DREB1A transgene during exposure to dehydration, salt, and low-temperature stress. Thus, strong stress tolerance was accomplished without significant growth retardation under control conditions. Indeed, the growth and seed production of these plants was similar to wild-type plants under normal growing conditions. *rd29A*:DREB1A plants are more tolerant to stress than the 35S:DREB1A plants. As the *rd29A* gene is one of the target genes of the DREB1A protein, the *rd29A* promoter is highly suited to the tissue-specific expression of DREB1A. In *rd29A*:DREB1A plants, the target gene products appear to accumulate in the same tissues that express the stress-inducible genes during stress conditions. These results indicate that transgenic expression of the DREB1A cDNA from the *rd29A* promoter should be quite useful for improving drought, salt, and

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freezing-stress tolerance in plants. Previously, we showed that the DRE also functions in stress response in tobacco plants[14,19], which suggests the existence of similar regulatory systems in tobacco and other crop plants. DRE-related motifs have been reported in the promoter region of cold-inducible *Brassica napus* and wheat genes[35,36]. These observations suggest that both the DREB1A cDNA and the *rd29A* promoter can be used to improve the dehydration, salt, and freezing tolerance of agriculturally important crops by gene transfer.

Experimental protocol

Overexpression of *DREB1A* in transgenic plants. The 35S:DREB1A plasmid was constructed as described previously[20]. To construct *rd29A*:DREB1A, a *Bam*HI fragment of the *DREB1A* cDNA was cloned into the *Bam*HI site of the pBI29APNot. pBI29APNot was constructed by ligation of the *Hind*III fragment of the *rd29A* promoter (-861 to +63) into the *Hind*III site of pBI101 (Clontech, Palo Alto, CA). The *Hind*III fragment of the *rd29A* promoter was amplified by PCR with the primers, 5′-AAGCTTGCCATAGATGCAATTAATC-3′ and 5′-AGCTTTTGGAAAGATTTTTTTCTTTCCAA-3′. The resulting plasmid was digested with *Sma*I and *Sac*I to delete the β-glucuronidase coding region, and ligated to a *Sma*I-*Not*I-*Sac*I polylinker (Takara, Tokyo, Japan). The constructs were introduced into *Agrobacterium tumefaciens* C58 as described previously[19]. The *Arabidopsis* plants selected for transformation were grown under continuous illumination in 9 cm pots at approximately 2500 lux and 22°C for 6 weeks. Plants were transformed by the vacuum infiltration method[22].

Phenotypes of the transgenic plants. The transgenic 35S:DREB1A and *rd29A*:DREB1A plants, which were grown on germination medium (GM) agar plates containing kanamycin (30 mg/L) for 7 days, were transferred to 9 cm pots filled with a 1:1 mixture of perlite and vermiculite, then were watered with 1000-fold diluted Hyponex (Osaka, Japan). They were photographed after the numbers of days indicated in Figures 2 and 4.

RNA gel blot analysis. *Arabidopsis* was grown on GM agar plates for 3 weeks and exposed to dehydration, high-salt, cold-stress, and ABA treatments, as described previously[19]. The plants were subjected to stress treatments for 5 h and then frozen in liquid nitrogen for further analyses. Isolation of total RNA and northern blot hybridization were performed as described previously[19]. The DNA fragments of the full-length *DREB1A*, *RD17*, *ERD10*, *AtP5CS*, *ERD1*, and *RD22* cDNAs and the 3′-terminal specific DNA fragments of *rd29A* and *rd29B* were used as probes. Probes for the *kin1*, *cor6.6*, and *cor15a* genes were obtained by PCR from cDNAs prepared from ABA-treated *Arabidopsis* plants.

Freezing, drought, and high-salt stress tolerance of the transgenic plants. Plants were grown in 9 cm pots filled with a 1:1 mixture of perlite and vermiculite. They were grown under continuous illumination of approximately 2500 lux at 22°C. Separate samples of the 3-week-old plants were exposed to freezing and drought stresses. Freezing stress was conducted by exposing the plants to -6°C temperatures for 2 days, then returning to 22°C for 5 days. Drought stress was conducted by withholding water for 2 weeks. High-salt stress was created by soaking plants that were grown on agar plates and gently pulled out of the growing medium in 600 mM NaCl solution for 2 h. The plants were then transferred to pots under normal growing conditions for 3 weeks. The numbers of plants that survived and continued to grow were counted. The statistical significance of the values was determined using chi-squared test.

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Localization and Characterization of Two Novel Genes Encoding Stereospecific Dioxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in *Delftia acidovorans* MC1

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Two novel genes, *rdpA* and *sdpA*, encoding the enantiospecific α-ketoglutarate dependent dioxygenases catalyzing *R,S*-dichlorprop cleavage in *Delftia acidovorans* MC1 were identified. Significant similarities to other known genes were not detected, but their deduced amino acid sequences were similar to those of other α-ketoglutarate dioxygenases. RdpA showed 35% identity with TauD of *Pseudomonas aeruginosa*, and SdpA showed 37% identity with TfdA of *Ralstonia eutropha* JMP134. The functionally important amino acid sequence motif $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R/K$, which is highly conserved in group II α-ketoglutarate-dependent dioxygenases, was present in both dichlorprop-cleaving enzymes. Transposon mutagenesis of *rdpA* inactivated *R*-dichlorprop cleavage, indicating that it was a single-copy gene. Both *rdpA* and *sdpA* were located on the plasmid pMC1 that also carries the lower pathway genes. Sequencing of a 25.8-kb fragment showed that the dioxygenase genes were separated by a 13.6-kb region mainly comprising a Tn*501*-like transposon. Furthermore, two copies of a sequence similar to IS*91*-like elements were identified. Hybridization studies comparing the wild-type plasmid and that of the mutant unable to cleave dichlorprop showed that *rdpA* and *sdpA* were deleted, whereas the lower pathway genes were unaffected, and that deletion may be caused by genetic rearrangements of the IS*91*-like elements. Two other dichlorprop-degrading bacterial strains, *Rhodoferax* sp. strain P230 and *Sphingobium herbicidovorans* MH, were shown to carry *rdpA* genes of high similarity to *rdpA* from strain MC1, but *sdpA* was not detected. This suggested that *rdpA* gene products are involved in the degradation of *R*-dichlorprop in these strains.

Chlorinated phenoxyalkanoic acids such as 2,4-dichlorophenoxyacetate (2,4-D), 2-methyl-4-chlorophenoxyacetate (MCPA), 2(2,4-dichlorophenoxy)propionate (dichlorprop), and 2(2-methyl-4-chlorophenoxy)propionate (mecoprop) have been widely used as broad leaf herbicides since their introduction in the 1940s. The ability to degrade the phenoxyacetate herbicides is widespread among bacteria (16). In contrast, breakdown of the structurally very similar chiral phenoxypropionate derivatives is far less widespread, and only few strains able to mineralize these compounds have been described (9, 21, 30, 35, 42, 50).

Degradation of phenoxyalkanoate herbicides is initiated by the etherolytic cleavage of the side chain yielding the respective phenol (Fig. 1). Subsequently, conversion into intermediates of the tricarboxylic acid cycle takes place via the modified *ortho*-pathway (16). Side chain cleaving specificity is crucial and determines the range of substrates that can be mineralized by a given microorganism.

Thus far, two types of 2,4-D-cleaving enzymes have been described. In *Ralstonia eutropha* JMP134, 2,4-D cleavage is catalyzed by a well-studied Fe(II) and α-ketoglutarate (αKG)-dependent dioxygenase, which is encoded by *tfdA* (13, 20, 39, 45, 59). The presence of the functionally essential motif $HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R/K$ indicates that TfdA is related to several other (referred to as group II) αKG-dependent dioxygenases (18). Recently, a three-dimensional model of the active site was proposed (10) on the basis of predictions made from site-directed mutagenesis studies (20) and secondary structure predictions (10). The identity of the suggested substrate-binding residues was confirmed by site-directed mutagenesis and inactivation studies (8). The *tfdA* genes are widespread among bacteria (17, 31, 33, 36, 39, 42, 47, 53, 54, 55, 56), also among strains unable to degrade 2,4-D (19). Recently, several α-proteobacterial strains were shown to carry *tfdA*α genes, which are *tfdA*-like, showing between 46 and 60% nucleotide identity to the canonical *tfdA* genes and forming a phylogenetic clade distinct from *tfdA* (22, 23). Three other genes, clustered as *cadABC*, were found to encode a second type of 2,4-D-cleaving enzyme, a putative monooxygenase responsible for 2,4-D cleavage in *Bradyrhizobium* sp. strain HW13 (24).

Studies on *Sphingobium* (formerly *Sphingomonas* [48]) *herbicidovorans* MH and *Delftia acidovorans* MC1 indicated that enantiospecific dichlorprop and mecoprop cleavage is catalyzed by Fe(II)- and αKG-dependent dioxygenases (35, 38). The enzymes have been biochemically characterized (34, 38, 57, 58), but no data regarding the genetic background are yet available. Thus far, the dioxygenases have been assumed to be *tfdA* gene products (39), because some dichlorprop- and mecoprop-degrading strains carry *tfdA* genes (36, 39) and cell extracts of *R. eutropha* strain JMP134 showed some specificity

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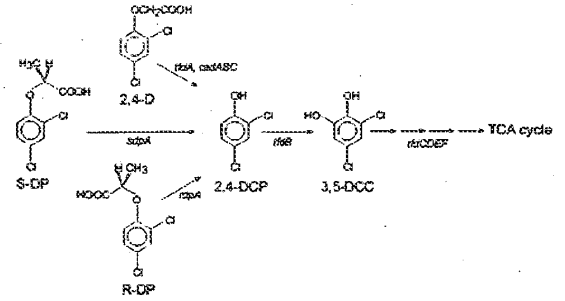


FIG. 1. Modified *ortho*-pathway for the degradation of chlorinated phenoxyalkanoate herbicides. 2,4-D, 2,4-dichlorophenoxyacetate; *R*-DP and *S*-DP, *R*- and *S*-enantiomers of 2(2,4-dichlorophenoxy)propionate, respectively; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; TCA, tricarboxylate.

toward cleaving *S*-dichlorprop (39). However, no activity for *S*-mecoprop or the *R*-enantiomers was detected in strain JMP134, and the organism was unable to grow on phenoxypropionate herbicides. Recently, available N-terminal and internal amino acid sequences of the enantiospecific dioxygenases from *D. acidovorans* MC1 showed only low similarities to TfdA enzymes (57), and *tfdA* genes were not detected in this strain (36). This suggested that genes other than *tfdA* are involved in dichlorprop degradation in strain MC1.

In *D. acidovorans* MC1 the first step in dichlorprop degradation was found to be highly unstable and easily lost under nonselective conditions (36). Loss of dichlorprop-cleaving activity was correlated with a deletion in the plasmid pMC1, and it was therefore hypothesized that the deleted fragment contains the genes encoding dichlorprop cleavage (36).

Based on the recently available peptide sequences of the *R*- and *S*-dichlorprop-cleaving dioxygenases, we investigated the genetic background of dichlorprop cleavage in *D. acidovorans* MC1. Two novel genes encoding the *R*- and *S*-dichlorprop-cleaving dioxygenases in *D. acidovorans* MC1 were identified and localized. The phylogenetic relationship of the *R*,*S*-dichlorprop-cleaving dioxygenases to other α-ketoglutarate-dependent dioxygenases was analyzed. The organization of these genes within the genome of strain MC1, as well as in a mutant deficient in dichlorprop cleavage, was investigated with regard to implications for the instability of this trait.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *D. acidovorans* MC1 and *Rhodoferax* sp. strain P230, isolated from contaminated building rubble (9, 35), *D. acidovorans* MC1-DP$^-$ (a mutant of strain MC1 arisen by spontaneous loss of dichlorprop-cleaving ability), and *S. herbicidovorans* MH, isolated from soil (21), were all grown on MM agar (36) containing 0.5 mM dichlorprop at 30°C. *Escherichia coli* TOP10F' (pCR2.1; Invitrogen) was used for cloning PCR products. The cosmid library was constructed in *E. coli* XL1-Blue MR (Stratagene). *E. coli* XL1-Blue MRF' (Stratagene) was used for maintaining pScosPA1. The vector pScosPA1 is a new broad-host-range cosmid identical to pSBC1 (43) except for a gentamicin resistance instead of the tetracycline resistance (E. Mahenthiralingam, unpublished data). A shotgun library of a cosmid was constructed by using the vector pcDNA2.1 (Invitrogen). *E. coli* S17-1 (pAG408::*gfp* [46]) was used in transposon mutagenesis experiments. *E. coli* strains were cultivated on Luria-Bertani (LB) agar at 37°C with the addition of appropriate antibiotics. Overnight cultures were prepared in liquid LB medium containing appropriate antibiotics at 37°C (*E. coli* strains) or 30°C (dichlorprop-degrading strains) on a rotary shaker. Antibiotics and dichlorprop were filter sterilized through a 0.2-μm-pore-size Minisart cellulose acetate filter (Sartorius) and added at a concentration of 100 μg/ml.

Mercury resistance was examined on LB agar plates containing 0.1 mM $HgCl_2$ incubated at 30°C for 48 h. *R. eutropha* JMP134(pJP4) and *R. eutropha* JM222 were used as positive and negative controls, respectively.

Construction of gene libraries. A genomic library of *D. acidovorans* MC1 was constructed by using cosmid pScosPA1, as described elsewhere (43). The average insert size of library clones was 34 kb. A total of 2,592 clones were picked into 96-well plates. After overnight growth 8% (vol/vol) dimethyl formamide was added, and the cosmid library was stored at −80°C.

A shotgun library of cosmid pScos-R5 was constructed in pcDNA2.1 according to the method of Buchrieser et al. (4) by using cosmid DNA isolated as described elsewhere (2, 3).

Transposon mutagenesis. The transposon of the suicide vector pAG408, a miniTn*5* derivative, carries a promoterless *gfp* gene (46) and was used for transposon mutagenesis of strain MC1. Mutagenesis was performed according to standard procedures (40). Overnight cultures of the donor and the recipient grown in LB medium supplemented with antibiotics (donor) were centrifuged, washed, and resuspended in LB medium to a final optical density at 600 nm (OD_{600}) of 1.0. Equal volumes of the donor and the recipient were mixed, and 100 μl of the mixture was plated out on LB agar, followed by incubation at 30°C for 6 h. The bacterial lawn was then washed from the plates with MM medium. Dilution series were prepared in MM and 100 μl of the 10^{-3} to the 10^{-5} dilutions was plated on selective MM agar plates containing 100 μg of gentamicin/ml, 27.8 mM fructose, and 0.5 mM dichlorprop. After incubation at 30°C for 6 days green fluorescing mutants were isolated and tested for dichlorprop degradation by high-pressure liquid chromatography as described elsewhere (60). In mutant clones able to degrade one of the enantiomers but not the other the region flanking the transposon was PCR amplified and sequenced.

DNA amplification. Degenerate primers (Table 1) were designed from N-terminal and internal peptide sequences of the enantiospecific dioxygenases of *D. acidovorans* MC1 (57) for PCR amplification of *rdpA* and *sdpA*. According to preliminary alignments of these peptide sequences with TfdA and TauD, the expected PCR fragment sizes were ca. 350 bp (*R*-dichlorprop dioxygenase gene) and 540 bp (*S*-dichlorprop dioxygenase gene). All primers were synthesized by MWG Biotech. Total genomic DNA used as a template was prepared from colonies grown on LB agar as described elsewhere (25). PCR was performed by using the *Taq* PCR Mastermix kit (Qiagen) and a PTC-200 thermal cycler (MJ Research). First, 100 pmol of each degenerate primer was added to 25 μl of PCR sample. The temperature gradient program involved an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 50 to 70°C for 45 s, and 72°C for 2 min, with a final elongation step of 72°C for 6 min. Amplification of an *rdpA* gene fragment with the primers rdp_fG and rdp_r involved an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 1 min, with a final elongation step of 72°C for 6 min. Amplification of an *sdpA* gene fragment with the primers sdp_fT and sdp_rA was the same as for *rdpA* amplification, except that the annealing step was performed at 60°C and that elongation lasted 90 s.

The *rdpA* genes from *S. herbicidovorans* MH and *Rhodoferax* sp. strain P230 were amplified with primers rdp_forw (5′-atg gta ggt ctc aaA TGC ATG CTG CAC TGT CCC CC-3′; lowercase letters denote a linker sequence) and rdp_rev (5′-atg gta ggt ctc agc gct CTA GCG CGC CGG GCG CAC G-3′). Amplification was the same as for the *rdpA* fragment except for the annealing step being performed at 60°C for 30 s.

Regions flanking Tn*5* transposon insertion sites were amplified by ST-PCR (6). To each sample either Tn*5*_outF (5′-GGC AGA TCT GAT CAA GAG-3′ [27]) annealing to the I end of the transposon or Tn*5*_outR (5′-CCG CAC TTG TGT ATA AGA GTC-3′) designed from the O end of the transposon were added as specific primers. Either of the following FP primers (44) was added as a random primer: FP-1, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ GGC CT-3′; FP-2, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ GCG CT-3′; FP-3, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ CCG GT-3′; and FP-4, 5′-AGT TCA AGC TTG TCC AGG AAT TC(N)$_7$ CGC GT-3′.

PCR products for the use as hybridization probes were generated as follows: the *mer* operon was partially amplified with primers mer_f (5′-CAC CAG TTC AGA CAG CAC G-3′) and mer_r (5′-ACC GGT GAA ACC AAG TCC-3′) amplifying a 1,450-bp fragment. The IS*91*-like element was partially amplified by using the primers IS_f (5′-ATG TCA GCC ACT TCC AAG C-3′) and IS_r (5′-GGA GGC AGT ATG CAT TTG G-3′) amplifying a 1,622-bp fragment. The PCR program for both fragments involved an initial denaturation step of 94°C for 3 min, followed by 36 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min, with a final elongation step of 72°C for 6 min. Amplification of *tfdB*, *tfdC*, and

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TABLE 1. Degenerate primers designed for the amplification of *rdpA* and *sdpA*

Peptide or primer	Sequence[a]	Position[b]
RDP_internal sequence 2	W E T L S P T M Q A T I E G L N	162–177
rdp_r	3′-AC GTY CGN TGN TAI CTY CC-5′	
RDP_internal sequence 3	N E I L D A F H T Y Q V I Y F P	43–58
rdp_fA[c]	5′-GCI TTY CAY ACN TAY CAA GT-3′	
rdp_fG[c]	5′-GCI TTY CAY ACN TAY CAG GT-3′	
SDP_N terminus	M Q T T L Q I T P T G A T L G A	1–16
sdp_fC[c]	5′-ATG CAR ACI ACI CTG CAR ATC AC-3′	
sdp_fT[c]	5′-ATG CAR ACI ACI CTG CAR ATT AC-3′	
SDP_internal sequence 1	L G H V Q Q A G S A Y I	193–204
sdp_rA[c]	3′-GTA CAN GTY GTY CGN CC-5′	
sdp_rG[c]	3′-GTA CAN GTY GTY CGN CC-5′	

[a] As described previously (57). IUPAC ambiguity codes: R = A or G, Y = C or T, N = any base, I = inosine.
[b] Alignment is shown in Fig. 2.
[c] To avoid degeneracy near the 3′ end, two pools of primers were designed from some peptides (sequence differences are indicated in boldface).

tfdD gene fragments was performed as described in reference 35. Fragments of *tfdE* and *tfdF* were amplified according to the method of Hoffmann et al. (17).

Sequencing and sequence analysis. For sequencing, PCR products were ligated to pCR2.1 and transformed into *E. coli* TOP10F′ by using the TA cloning kit (Invitrogen). Cycle sequencing was performed by using the ABI Prism BigDye Terminator v2.0 cycle sequencing ready reaction kit (PE Applied Biosystems). M13 primers were used for amplifying and sequencing cloned PCR fragments. After amplification with M13 primers and electrophoretic separation, fragments were extracted with the E.Z.N.A gel extraction kit (Peqlab Biotechnologie). Sequencing reactions (total volume, 10 μl) contained 2 μl of premix, 10 pmol primer, and 10 to 50 ng of template DNA. Primer walking on cosmid DNA was performed with primers designed from the sequences of the *rdpA* and *sdpA* PCR products. Sequencing reactions (total volume, 20 μl) contained 6 μl of premix, 20 pmol of primer, and 500 ng of cosmid DNA. After 25 cycles of 96°C for 30 s, 55°C for 15 s, and 60°C for 4 min, the DNA was precipitated by adding 80 μl of H_2O, 10 μl of 3 M sodium acetate (pH 4.6), and 250 μl of ethanol; washed with 70% ethanol; vacuum dried; and redissolved in 20 μl of template suppression reagent (PE Applied Biosystems). Sequencing from cosmid DNA involved an additional purification step prior to the ethanol precipitation by using Centri-Sep spin columns (Princeton Separations). After denaturation at 90°C/2 min the sample was chilled on ice. Electrophoresis, data collection, and analysis were carried out on an ABI Prism 310 genetic analyzer (PE Applied Biosystems). Both strands were sequenced. Sequencing data were analyzed with the Sequence Navigator software, contigs were assembled by using the Autoassembler software (PE Applied Biosystems), and the BLASTn and BLASTx programs (version 2.2.2. [1]) were used to search for similar sequences in nucleotide and protein sequence databases. Sequences were aligned by using the programs CLUSTAL W (51) or MultAlign (7). Phylogenetic analysis was performed by using the protml tool of the ARB package (29) when the maximum-likelihood method was applied and MEGA2.1 (26) when trees were constructed with the neighbor-joining or maximum-parsimony method.

Recombinant pcDNA2.1 DNA was isolated as described elsewhere (2, 3). A Perkin-Elmer 9700 thermocycler was used for cycle sequencing. Sequencing reactions (total volume, 10 μl) contained ca. 300 ng of template, 1.5 μl of ABI Prism BigDye Terminator v1.1 Cycle Sequencing RR-100 mix, 1.2 μl of 5× sequencing buffer, and 2.4 pmol of primer T7 or M13. The sequencing program involved an initial denaturation step (94°C for 60 s), followed by 70 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reactions were precipitated with 40 μl of 76% ethanol for 30 min, washed with 70% ethanol, and resuspended in 10 μl of 0.3 mM EDTA buffer. Electrophoresis, data collection, and analysis were performed by using an ABI Prism 3700 genetic analyzer (PE Applied Biosystems). Automatic sequence assembly was carried out by using PHRED (11, 12), PHRAP (P. Green, unpublished data), and the Autoassembler software. When gaps were closed between contigs, cosmid DNA was used as a template and custom-made oligonucleotides were added as primers. The sequence was edited by using CONSED (15) and annotated by using Vector NTI Advance (InforMax). Similarity searches were carried out as mentioned above.

DNA-DNA hybridization. Screening of the cosmid library was done by replica plating 12 96-well grids onto positively charged nylon membranes (Roche) that had been placed on selective agar. Cells of strain MC1 (positive control) and *E. coli* XL1-Blue MRF′ (pScosPA1) (negative control), as well as strains MH and P230, were smeared onto the nylon membrane placed on LB agar. After overnight growth, DNA was immobilized onto the membranes according to the manufacturer's guidelines.

Genomic DNA of strain MC1 was isolated as described by Sokol et al. (43). The low-copy plasmid pMC1 was isolated with a Nucleobond AX100 kit (Macherey-Nagel). Plasmid and genomic DNA were PstI digested according to standard procedures (40), separated electrophoretically in 0.8% agarose, blotted onto positively charged nylon membrane according to the manufacturer's guidelines, and cross-linked by UV irradiation for 1 min.

Cosmid and plasmid DNA was isolated from 20 ml of LB medium by using the Nucleobond AX100 kit (Macherey-Nagel). Next, 5 μl of cosmid DNA was applied to positively charged nylon membrane and cross-linked by UV irradiation for 1 min. then, 100 μl of plasmid DNA was PstI or EcoRI/XmaI digested according to standard procedures (40), ethanol precipitated, separated, blotted, and UV cross-linked as described above.

PCR fragments were digoxigenin labeled by using the DIG DNA labeling kit (Roche). For hybridization and immunodetection, the DIG Easy Hyb Wash and Block buffer set and the DIG nucleic acid detection kit (Roche) were used according to the manufacturer's guidelines. Hybridizations were performed at 50°C (*tfdC*, *tfdD*, *tfdE*, and *tfdF* probes), 55°C (*rdpA* probe), 60°C (probes against *tfdB*, the *mer* operon, and the IS*91* element), or 62°C (*sdpA* probe). Stripping was carried out according to the manufacturer's instructions.

Nucleotide accession numbers. The nucleotide sequences determined in the present study were deposited in the GenBank, EMBL, and DDBJ databases under accession numbers AY327575, AF516751, and AF516752.

RESULTS

Identification, sequencing, and analysis of dichlorprop-cleaving dioxygenase genes in *D. acidovorans* MC1. Degenerate primers were designed from N-terminal and internal peptide sequences of both enantiospecific dioxygenases of *D. acidovorans* MC1. Primers with a degeneracy near the 3′ end proved to be nonspecific; therefore, two pools of primers were synthesized to avoid degeneracy at such positions (Table 1). PCR with the primer pairs rdp_fG-rdp_r (*rdpA*-specific primers) and sdp_fT-sdp_rA (*sdpA*-specific primers) yielded fragments of 339 and 532 bp, respectively, whereas all other primer combinations did not produce specific fragments. The two PCR fragments were cloned and sequenced. The deduced amino acid sequences of both fragments contained sequences at their N and C termini, respectively, that correctly corresponded to the peptide sequences used for designing the prim-

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TABLE 2. Sequence identities of the dichlorprop-cleaving enzymes RdpA and SdpA to other group II αKG-dependent dioxygenases

α-KG dioxygenase[a]	Strain, accession no.	% Identities[b] to	
		RdpA	SdpA
TfdA	*Ralstonia eutropha*, A27082	32/279	37/244
TauD	*Pseudomonas aeruginosa*, NP_252624	35/279	27/258
AtsK	*Pseudomonas putida*, AAD31784	31/296	31/296
YSD	*Saccharomyces cerevisiae*, Q12358	29/279	27/304
PvcB	*Pseudomonas aeruginosa*, AAC21672	20/298	21/277
SyrI	*Pseudomonas syringea*, AAB63253	17/323	18/308
CarC	*Pectobacterium carotovorum*, AAD38231	16/285	17/275
γ-BBH	*Pseudomonas* sp. strain AK-1, P80193	16/309	17/291
CS2	*Streptomyces clavuligerus*, AAA26723	16/208	17/275

[a] TfdA, 2,4-D/αKG dioxygenase; TauD, taurine/αKG dioxygenase; AtsK, putative αKG-dependent alkylsulfatase; YSD, sulfonate/αKG dioxygenase; PvcB, pyoverdine biosynthesis enzyme; SyrI, syringomycin biosynthesis enzyme I; CarC, carbapenem biosynthesis enzyme C; γ-BBH, γ-butyrobetaine hydroxylase; CS2, clavaminate synthase 2.

[b] That is, the percent sequence identity over the total number of amino acids examined.

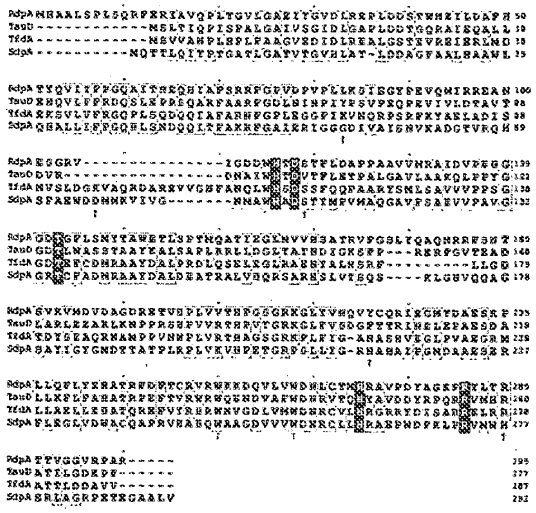


FIG. 2. Sequence alignment of RdpA and SdpA with other group II αKG-dependent dioxygenases. TfdA, *R. eutropha* 2,4-D/αKG dioxygenase (accession no. A27082); TauD, *P. aeruginosa* taurine/αKG dioxygenase (accession no. NP_252624). Conserved Fe(II)- and αKG-binding residues are shaded in black; other conserved regions are shaded in gray. Black arrows indicate 2,4-D binding residues in TfdA; gray arrows indicate residues undergoing posttranslational hydroxylation in TauD or TfdA. Amino acid positions numbers are shown to the right of each line. The alignment was created with CLUSTAL W.

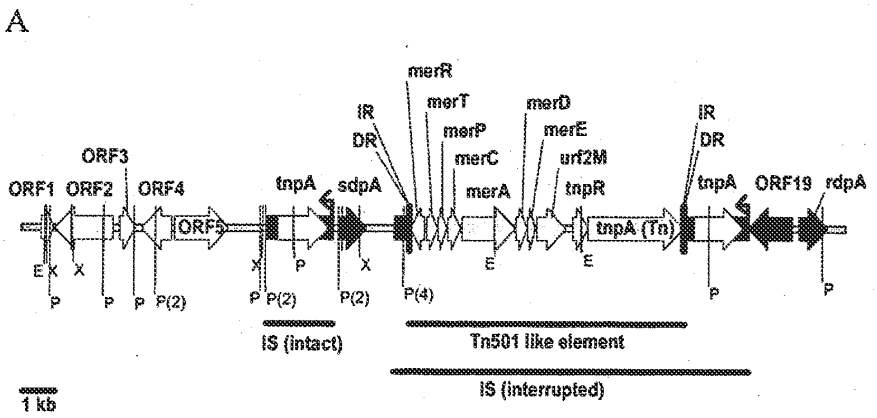
ers. In screens of a genomic cosmid library of strain MC1 10 of 1,152 clones hybridized with the *rdpA* gene probe. Of these 10, 4 hybridized with the *sdpA* gene probe, but PCR with the *sdpA*-specific primers yielded a product with only 1 of these 4 clones, designated pScos-R5. Both dioxygenase genes were then completely sequenced from this clone. The coding regions of the *R*-dichlorprop and *S*-dichlorprop dioxygenase genes were 888 and 879 bp, respectively. No significant sequence similarities to other known genes were found searching nucleotide databases. However, the deduced amino acid sequences of RdpA and SdpA both shared significant similarities to other αKG-dependent dioxygenases ranging between 37 and 16% (Table 2). RdpA and SdpA shared 26% sequence identities. Furthermore, similarities to a number of putative dioxygenases belonging to the genera *Pseudomonas*, *Yersinia*, *Ralstonia*, *Streptomyces*, *Bordetella*, *Bradyrhizobium*, *Azotobacter*, *Shigella*, *Chromobacterium*, *Burkholderia*, and *Mycobacterium* were found and ranged between 31 and 16%.

The amino acid sequences of both dioxygenases were analyzed on the basis of the alignment shown in Fig. 2. It shows that an internal sequence tentatively aligned to positions 257 to 287 of TfdA by Westendorf et al. (57), comprises positions 85 to 131 of RdpA.

Transposon mutagenesis. Of 959 green fluorescing transposon mutants, 2 were found to carry the transposon insertion in *rdpA*. In clone MC1-T270, the insertion was located after bp 627; in clone MC1-T647, the mini-Tn*5* had inserted after bp 814. Both mutants had lost the ability to cleave *R*-dichlorprop but were still able to grow on the *S*-enantiomer.

Organization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1. The 25,768-bp sequence of the genomic fragment from strain MC1 containing *rdpA* and *sdpA* in cosmid pScos-R5 is represented in Fig. 3A. The assembly was verified by PCR of selected regions (data not shown). The *rdpA* and *sdpA* genes were separated by 13.6 kb, which mainly comprised an 8.7-kb putative transposon, organized in a way characteristic for the Tn*501* subgroup of the Tn*3* transposon family (28). It was flanked by 38-bp inverted repeats that were flanked by 5-bp direct repeats generated during insertion. The open reading frames (ORFs) for the putative transposase and resolvase genes *tnpA* and *tnpR* had an outward direction with the ORF for *tnpA* proceeding 5 bp into the inverted repeat. The *res* site, at which cointegrate resolution during transposition takes place, was located upstream of *tnpR*. The remaining part of the transposon was occupied by a mercury resistance gene cluster. The *merR* gene had a direction divergent from the *merTP CADE* operon. A putative *urf2M* was located adjacent to the *mer* operon. The putative Tn*501* transposon showed 81% nucleotide sequence identity to a putative Tn*3* family transposon of *Nitrosomonas europaea* ATCC 19718 (accession no. BX321858.1). The deduced amino acid sequences of the identified ORFs showed between 82 and 97% identity to proteins involved in transposition and mercury resistance (Table 3). The functionality and mobility of this transposon were not tested, but strain MC1 was found to be resistant to 0.1 mM $HgCl_2$. Mercury resistance was not lost when dichlorprop-cleaving activity was lost, but these mutants were still able to grow in the presence of 0.1 mM $HgCl_2$. In Southern hybridizations the *mer* operon of the plasmid was localized on a 4.4-kb fragment of pMC1 (Fig. 4C). This copy was deleted in the plasmid of the mutant MC1-DP$^-$. However, in hybridizations against genomic DNA, another signal at 2.8 kb was detected that was present in the wild-type strain as well as in strain MC1-DP$^-$ (data not shown), suggesting that strain MC1 carries a second copy of the *mer* operon on the chromosome that is not lost when the dichlorprop-cleaving ability is lost. It is possible that the chromosomal copy of the *mer* operon confers mercury resistance to strains MC1 and MC1-DP$^-$, whereas the copy on the plasmid may lack functionality.

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B

IS*91* family: AnnnATAGGAAnTTnAAnnnn

putative IS*91* element strain MC1: ccagATAGGAAaTTcAAccgc

FIG. 3. Organization of *rdpA* and *sdpA* within the genome of strain MC1. (A) Genetic map of the genomic fragment containing *rdpA* and *sdpA*. For ORFs showing a high degree of similarity to other genes (>80% identity of the deduced amino acid sequence) the respective names are given. The gene designations are as follows: *tnpA*/*tnpA*(Tn), putative transposases; *sdpA*, *S*-dichlorprop/α-ketoglutarate dioxygenase; *merR*, putative mercury resistance operon transcriptional regulator; *merT*, *merP* and *merC*, putative mercury ion transport genes; *merA*, putative mercury reductase; *merD*, putative secondary regulator; *merE* and *urf2M*, conserved ORF of unknown function; *tnpR*, putative site-specific resolvase; and *rdpA*, *R*-dichlorprop/α-ketoglutarate dioxygenase. ORF1 through ORF5 and ORF19 show significant similarities to genes encoding the proteins as follows: ORF1, carboxymuconolactone decarboxylase; ORF2, potassium/proton antiporter; ORF3, bacterioferritin; ORF4, LysR type regulator; ORF5, dihydroxyacid dehydratase; and ORF19, MFS transporter. The positions of the following features are indicated: IR, 38-bp inverted repeat flanking the Tn*501*-like transposon, DR, 5-bp direct repeat. Two putative IS*91*-like elements are shown as black boxes; the positions of the *ori*IS are indicated as arrows above the sequence. The right IS*91*-like element is interrupted by a Tn*501*-like transposon. Its left partial sequence is not drawn to scale. Single letters denote the following restriction sites: E, EcoRI; P, PstI; and X, XmaI. In the case of multiple PstI restriction sites within less than 100 bp, only the most left site is given and the number of sites is indicated in brackets. (B) Comparison of the sequence at the right end of the putative IS elements with the IS*91* family *ori*IS consensus sequence. Conserved nucleotides are shown in bold uppercase letters.

Furthermore, two copies of a putative IS element resembling the IS*91*/IS*801* family of insertion elements were identified. Members of this family of IS elements do not contain inverted repeats and do not create duplications of the target sequence (14). Insertion of this family of elements is site specific, and replication utilizes the rolling circle mechanism; therefore, an *ori*IS, the origin of replication, and a *ter*IS, the termination sequence, are found at either end of these IS elements (14). The two copies of the putative IS elements in strain MC1 were essentially identical except for a 9-bp deletion and a 47-bp deletion in the left, intact copy. The element next to ORF19 was interrupted by the Tn*501*-like element (Fig. 3A). The elements were 2,037 bp (intact copy) and 2,084 bp (interrupted copy) and contained an IS*801*-like transposase gene showing 99% sequence identity to the homologues on the atrazine catabolic plasmid pADP-1 (Table 3 [32]) and a transposase associated with chlorobenzene degradation genes in *Ralstonia* sp. strain JS705 (37). At the right end of both elements a sequence was identified, that matched the consensus sequence for origin of replication *ori*IS of this group of insertion elements (49) in all but the first nucleotide (Fig. 3B). Neither the termination sequence *ter*IS nor the tetramers GTTC/CTTG for which IS*91* family elements show site specificity were detected.

ORF19, located upstream of *rdpA* showed significant similarities to aromatic compound major facilitator superfamily (MFS) transporters. A 29% identity to the 2,4-D transporters CadK of *Bradyrhizobium* sp. strain HW13 and TfdK of *Achromobacter xylosoxidans* subsp. *denitrificans* and *R. eutropha* JMP134 was detected. A putative Shine-Dalgarno sequence was found 7 bp upstream of the start codon.

Another five ORFs, ORF1 to ORF5, were identified at the left end side of the insert.

Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of the wild-type plasmid pMC1 with that of the mutant MC1-DP⁻. Southern hybridizations showed that both *rdpA* and *sdpA* were located on the

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TABLE 3. Localization and predicted function of ORFs in the genomic region of strain MC1 containing *rdpA* and *sdpA*

ORF	Coordinates (bp)	Coding strand[a]	Function of closest match	Source microorganism	% Amino acid identity[b]	No. of amino acids MC1/match[c]	Accession no.
1	574–966	d	Carboxymuconolactone decarboxylase family protein	*Shewanella oneidensis* MR-1	65 (81/124)	130/128	AAN53170.1
2	1606–2799	c	Glutathione-regulated potassium-proton antiporter	*Pseudomonas putida* KT2440	69 (385/556)	597/591	AEO16523.1
3	3018–3494	d	Putative bacterioferritin	*Delftia acidovorans*	70 (112/158)	158/158	CAC83620.1
4	3701–4642	c	Hypothetical protein, similar to LysR-type regulators	*Ralstonia metalliduran*s	51 (161/314)	313/360	ZP 00022545.1
5	4745–6438	d	Putative dihydroxy acid dehydratase	*Ralstonia solanacearum*	76 (420/555)	564/557	CAD16119.1
6	7981–9528	d	Putative transposase, similar to IS*801* transposase	*Pseudomonas* sp. strain ADP	99 (512/515)	515/515	AAK50272.1
7	9836–10714	d	2,4-D dioxygenase (TfdA)	*Ralstonia eutropha* JMP134	37 (92/249)	292/287	A27082
8	12107–12541	c	Mercury resistance regulatory protein (MerR)	*Enterobacter aerogenes*	97 (140/144)	144/144	CAA83896.1
9	12613–12963	d	Mercury uptake protein (MerT)	*Alcaligenes* sp.	96 (112/116)	116/116	CAA70196.1
10	12977–13251	d	Mercury uptake protein (MerP)	*Alcaligenes* sp.	94 (86/91)	91/91	CAA70197.1
11	13279–13701	d	Mercury uptake protein (MerC)	*Nitrosomonas europaea* ATCC 19718	86 (121/140)	140/140	CAD84751.1
12	13741–15426	d	Mercury ion reductase (MerA)	*Klebsiella pneumoniae*	88 (494/561)	561/561	AAM44226.1
13	15444–15809	d	Mercury resistance secondary regulatory protein (MerD)	*Pseudomonas* sp. strain ADP	95 (115/121)	121/121	AAK50284.1
14	15806–16042	d	Putative protein (MerE)	*Pseudomonas* sp. strain ADP	91 (71/78)	78/78	AAK50283.1
15	16093–17028	d	Putative protein, similar to Urf2M	*Ralstonia metalliduran*s	88 (274/310)	311/329	CAD97548.1
16	17245–17718	d	Resolvase (TnpR)	*Nitrosomonas europaea* ATCC 19718	98 (154/157)	158/186	CAD84747.1
17	17721–20690	d	Tn*3* family transposase (TnpA)	*Nitrosomonas europaea* ATCC 19718	95 (945/989)	989/989	CAD84746.1
18	21024–22571	d	Putative transposase, similar to IS*801* transposase	*Pseudomonas* sp. strain ADP	99 (511/515)	515/515	AAK50727.1
19	22752–24137	c	Putative aromatic compound MFS transporter	*Pseudomonas putida* KT2440	73 (318/433)	461/458	AAN69258.1
20	24330–25217	d	Taurine dioxygenase (TauD)	*Pseudomonas aeruginosa* PA01	35 (100/284)	295/279	NP_252624

[a] c, complementary strand; d, direct strand.
[b] Values in parentheses refer to numbers of identical amino acids over the total number examined.
[c] Number of amino acids in strain MC1 ORF/number in the closest match.

plasmid pMC1 (Fig. 4A). Distinct signals of the expected fragment sizes of 3.5 kb (*rdpA*) and 1.9 kb (*sdpA*) were observed with plasmid DNA (Fig. 4A, lane 1). Much fainter signals of the same fragment sizes were obtained with the genomic DNA, making visible the plasmid content of this. Loss of dichlorprop cleavage in strain MC1-DP$^-$ was associated with a deletion of both dioxygenase genes from pMC1-DP$^-$ (Fig. 4B). Furthermore, the *mer* operon and most of the numerous copies of the IS*91*-like elements were also deleted in the mutant MC1-DP$^-$ (Fig. 4C). In contrast, the lower pathway genes *tfdB-F* remained unaffected (data not shown).

Screening for *rdpA* and *sdpA* in other dichlorprop-degrading bacteria. Southern hybridizations with *rdpA* and *sdpA* probes showed that neither *S. herbicidovorans* MH nor *Rhodoferax* sp. strain P230 carried *sdpA*-like genes, but both strains gave signals with the *rdpA* gene probe (data not shown). The genes were PCR amplified and sequenced. The *rdpA* gene of strain P230 was identical to that of strain MC1; the one of strain MH contained one neutral base substitution.

DISCUSSION

Two novel genes encoding the enantiospecific dichlorprop and mecoprop cleaving dioxygenases RdpA and SdpA in *D. acidovorans* MC1 were identified. The characterization of genes shown to be involved in the cleavage of these chiral herbicides has not previously been reported. To date, *tfdA* genes or genes closely related in sequence to *tfdA* were proposed to be involved in *R*-dichlorprop cleavage in *Alcaligenes denitrificans* (39). This suggestion was based on the observations that a *tfdA* gene is present in *Alcaligenes denitrificans* and that dichlorprop cleavage is catalyzed by an αKG-dependent dioxygenase in this strain as well as in *S. herbicidovorans* MH (38). However, it was not supported by *tfdA* expression studies or amino acid sequences of the *R*-dichlorprop cleaving dioxygenase. The *rdpA* and *sdpA* genes of strain MC1 showed no significant nucleotide similarities to *tfdA*. However, the significant degree of identity of their deduced amino acid sequences to group II αKG-dependent dioxygenases and the distribution of these identities throughout the whole of the primary sequence (Fig. 2) supported the view that RdpA and SdpA are homologous to dioxygenases of this family. The functionally important motif $HX(D/E)X_{23\text{-}26}(T/S)X_{114\text{-}183}HX_{10\text{-}13}R/K$, which is conserved throughout group II αKG-dependent dioxygenases (18), is present in both RdpA and SdpA. In TfdA of *R. eutropha* it includes the three Fe(II)-binding residues His114, Asp116, and His263, and the two αKG-binding residues Thr141 and Arg274 (Fig. 2). All of these residues were conserved in RdpA and SdpA, representing the putative Fe(II) and αKG-binding residues in these enzymes. Arg278, His214, Ser117, Lys95, and Lys71 of TfdA are known to interact with 2,4-D (8, 10). Ser117 homologues were identified in both dichlorprop-cleaving enzymes. A homologue for Arg278 was present only in RdpA, one for His214 was found in SdpA. The tryptophan residues Trp126, Trp238, and Trp246 undergoing posttranslational hydroxylation in TauD and Trp113 undergoing posttranslational hydroxylation in TfdA form a hydrophobic cluster around the active site (10). In RdpA and SdpA all of these residues were conserved or substituted by other hydrophobic amino acids. The basis of the enantiospecificity of

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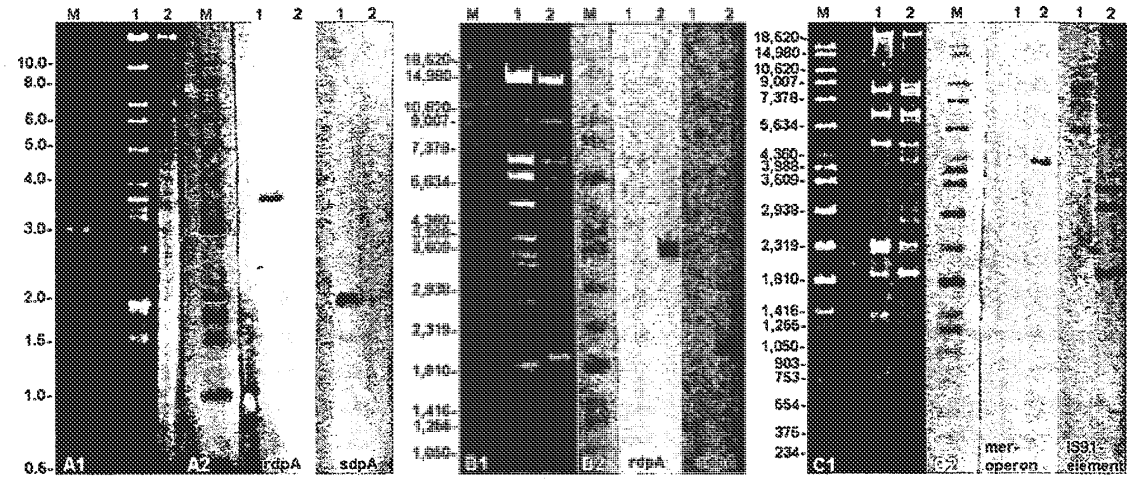


FIG. 4. Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of plasmid pMC1 with pMC1-DP⁻, the plasmid of mutant MC1-DP⁻. (A) Localization of *rdpA* and *sdpA* within the genome of strain MC1. Plasmid DNA (lane 1) and genomic DNA (lane 2) was digested with PstI, separated electrophoretically in 0.8% agarose (A1), and hybridized (A2). Gene probes are given at the bottom. (B and C) Comparison of plasmid pMC1 of the wild-type strain of MC1 (lane 2) with pMC1-DP⁻, the plasmid of the mutant unable to cleave dichlorprop (lane 1). PstI-digested plasmid DNA was separated electrophoretically in 0.8% agarose (B1) and hybridized against *rdpA* and *sdpA* gene probes (B2). EcoRI/XmaI-digested plasmid DNA was separated electrophoretically in 0.8% agarose (C1) and hybridized against probes for the *mer* operon and the IS*91* element (C2).

the dichlorprop-cleaving enzymes is not known. It is known that the tertiary structure of homologous proteins showing at least 20% identity of amino acid sequence is conserved (41). Among group II αKG dioxygenases a high degree of structural conservation was observed between clavaminate synthase and TauD, which share as little as 7.7% primary sequence identity (10). It is therefore likely that 2,4-D and dichlorprop-cleaving enzymes are structurally very similar and that substrate specificity is determined by only a few amino acids. Site-specific mutagenesis studies are needed to reveal the molecular basis of the substrate specificity and stereospecificity of RdpA and SdpA.

Phylogenetic trees calculated by different methods (data not shown) consistently grouped RdpA in one clade with TauD enzymes, but the assignment of SdpA could not be determined precisely. The primary sequence of SdpA most closely resembled that of TfdA and, like this dioxygenase, SdpA is characterized by a relatively broad substrate specificity (34). TfdA has greatest activity with 2,4-D but shows some activity toward *S*-mecoprop (38). SdpA shows greatest activity with the *S*-enantiomers of mecoprop and dichlorprop but has some activity toward 2,4-D (34). These observations suggested that SdpA is most closely related to 2,4-D-cleaving enzymes, which was also consistent with the position of SdpA in trees constructed without an outgroup. However, when PvcB was used as an outgroup SdpA was represented as a sister group of both the TfdA/TfdAα and the RdpA/TauD clades. At present, the database is too small to determine more precisely the phylogenetic relationship between SdpA and other members of the αKG dioxygenase family.

Transposon mutagenesis inactivation of *rdpA* indicated that it was a single-copy gene and gave a further proof of its functionality and role in strain MC1, besides previous characterization of RdpA and SdpA (34, 57, 58).

An *rdpA* gene identical to that of strain MC1 was identified in *Rhodoferax* sp. strain P230, and one nearly identical was found in *S. herbicidovorans* MH, but neither of these strains carried *sdpA* genes, as indicated by Southern hybridizations allowing ca. 20% mismatches. The *rdpA* genes could be involved in *R*-dichlorprop degradation in strains P230 and MH. Further investigations are necessary to study their role to determine whether *rdpA* genes are generally more widely distributed than *sdpA* genes, as these limited data suggest, and to identify the genes encoding the *S*-dichlorprop-cleaving dioxygenases of strains MH and P230.

In *S. herbicidovorans* MH, three transport proteins specific for 2,4-D, *R*-dichlorprop, and *S*-dichlorprop were identified (60). In strain MC1, ORF19 encodes a putative transport protein (Fig. 4 and Table 3). It was in close proximity to *rdpA*, and the significant similarity of the deduced protein sequence to TfdK and CadK suggested an involvement in *R*-dichlorprop transport. No other potential transporters were detected. Likewise, no genes encoding transcriptional regulators of dichlorprop cleavage were identified. This is in accordance with previous studies which found *rdpA* and *sdpA* to be constitutively expressed (36). ORF4, which shows significant similarities to LysR-type transcriptional regulators, probably regulates the expression of ORF5 rather than that of *sdpA* or *rdpA*.

The ability to degrade dichlorprop in *D. acidovorans* MC1 is spontaneously and irreversibly lost at high frequencies under nonselective conditions, whereas the degradation of dichlorophenol is a stable trait (36). This indicated that the ability to cleave the phenoxyalkanoate herbicides is unstable in strain MC1, which would be consistent with recent acquisition of the

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rdpA and *sdpA* genes. The localization and organization of the initial step in dichlorprop degradation imply that its evolution and recruitment took place independently of the lower pathway. Cases and de Lorenzo (5) postulated that degradative pathways for substrates only very recently found in the environment are often poorly regulated, probably reflecting an early step in the optimization of the corresponding metabolic route, and that biodegradative operons evolve from constitutive expression to substrate-responsive and metabolically controlled transcription. The evolution of the dichlorprop cleavage pathway in strain MC1 is an example supporting this hypothesis, since *rdpA* and *sdpA* are constitutively expressed, whereas the consecutive degradation of the intermediate dichlorophenol is inducible (36).

Previously, the loss of genes involved in etherolytic cleavage of dichlorprop was hypothesized to be caused by a deletion within plasmid pMC1, which occurs concomitantly with loss of dichlorprop cleavage (36). In the present study, both dioxygenase genes were shown to be located on the plasmid and to be deleted in the MC1-DP$^-$ mutant, proving this hypothesis to be correct. Moreover, the *mer* operon was deleted, and the copy number of the IS*91*-like element was found to be considerably reduced. This suggests that recombination between IS*91* elements could lead to the deletion of one or both dioxygenase genes and the Tn*501*-like transposon. Although IS*91*-like elements do not cause deletion of regions adjacent to them, a deletion of a genomic fragment flanked by IS*91*-like elements was observed in *Shigella flexneri* (52). In strain MC1, recombination between the interrupted IS element and the intact IS copy downstream of ORF5 would lead to the deletion of the region between the elements, including *sdpA*. Likewise, the IS elements may have played a role in the acquisition of dichlorprop cleavage in strain MC1. In *Ralstonia* sp. strain JS705 the *clc* genes are flanked by an intact and a partial copy of an insertion element highly similar to the ones identified in strain MC1. These IS elements were proposed to have captured the *clc* genes and made possible the gene transfer from *Ralstonia* sp. strain JS745 to strain JS705 (37). Characterization of the region downstream of *rdpA* and comparison of the MC1-DP$^-$ mutant genotype with the MC1 wild-type will further the understanding of processes having lead to the acquisition and causing instability of the degradative trait.

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Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CVMV) promoter

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Abstract

The cassava vein mosaic virus (CVMV) is a double stranded DNA virus which infects cassava plants (*Manihot esculenta* Crantz) and has been characterized as a plant pararetrovirus belonging to the caulimovirus subgroup. Two DNA fragments, CVP1 of 388 nucleotides from position −368 to + 20 and CVP2 of 511 nucleotides from position −443 to +72, were isolated from the viral genome and fused to the *uid*A reporter gene to test promoter expression. The transcription start site of the viral promoter was determined using RNA isolated from transgenic plants containing the CVMV promoter:*uid*A fusion gene. Both promoter fragments were able to cause high levels of gene expression in protoplasts isolated from cassava and tobacco cell suspensions. The expression pattern of the CVMV promoters was analyzed in transgenic tobacco and rice plants, and revealed that the GUS staining pattern was similar for each construct and in both plants. The two promoter fragments were active in all plant organs tested and in a variety of cell types, suggesting a near constitutive pattern of expression. In both tobacco and rice plants, GUS activity was highest in vascular elements, in leaf mesophyll cells, and in root tips.

Introduction

The cassava vein mosaic virus (CVMV) infects cassava plants in north-eastern regions of Brazil. Infected plants develop chlorosis along the veins which evolves later into a mosaic pattern. The circular double stranded DNA genome of the CVMV is encapsidated in 50–60 nm isometric particles which accumulate in inclusion bodies, in the cytoplasm of infected cells [28]. Based upon this information CVMV was listed as a putative pararetrovirus and a candidate member of the caulimovirus subgroup [23]. Recently the molecular characterization of the CVMV genome reported by Calvert *et al.* [10] showed an atypical genomic organization distinct from other characterized plant pararetroviruses [12]. The CVMV genome is 8158 nucleotides long and is organized in five open reading frames. Consensus sequences for coat protein, movement protein and replicase have been identified and support the classification of CVMV as a plant pararetrovirus. However, the putative coat protein domain is located upstream of the putative movement protein sequence in the same ORF, suggesting that they are transcribed as a polyprotein. This particular organization is unusual for both caulimoviruses and badnaviruses [32], the two pararetrovirus subgroups.

Several transcriptional promoters which are capable of causing high levels of gene expression in transgenic plants have been isolated from pararetrovirus genomes [8, 34, 37, 46]. During virus infection, these promoters direct the synthesis of genome-length RNAs which serve as templates for the reverse-transcriptase and as messenger RNAs for synthesis of viral proteins. The 35S promoter from the cauliflower mosaic virus (CaMV) has been widely used in chimeric gene constructs and is well characterized [4, 15, 37, 39]. Synergistic and additive interactions amongst the different *cis* elements upstream of the TATA box combine to confer a constitutive pattern of expression [5, 6, 7]. The 34S promoter from another caulimovirus, the fig-

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wort mosaic virus (FMV), is also constitutively active in transgenic plants and has been reported to be functionally analogous to the 35S promoter [46]. Promoters isolated from badnaviruses, unlike caulimovirus promoters, seem to be primilary active in vascular tissues. Indeed, it has been reported that the rice tungro bacilliform virus (RTBV) and *Commelina* yellow mottle virus (CoYMV) promoters direct phloem-specific gene expression in transgenic plants [8, 34, 51].

The present study was undertaken to identify the promoter region of the cassava vein mosaic virus and to determine its ability to promote gene expression in transgenic plants. Furthermore, we wanted to analyze the properties of the promoter of this atypical caulimovirus in comparison with those of others promoters isolated from plant pararetroviruses. In this report we describe the isolation of two CVMV promoter fragments and provide evidence of promoter activity using *uid*A gene fusion constructs. The strength of these promoters was evaluated in electroporated protoplasts of cassava and tobacco cell suspension cultures and the pattern of expression was characterized in stably transformed rice and tobacco plants. Evidence of promoter activity in cassava plants was provided by microbombardment of *in vitro* plantlets.

Materials and methods

Isolation of promoter fragments and plasmid constructions

Molecular techniques were carried out essentially as described by Sambrook *et al.* [44]. Nucleotide numbers refer to the cassava vein mosaic virus genome nucleotide sequence reported by Calvert *et al.* [10] as concurrently determined in this laboratory (unpublished). The original CVMV full length genomic clone was provided by Dr R.J. Shepherd (University of Kentucky). The clone was constructed by ligating the viral DNA cut at a unique *Bgl*II site to the *Bgl*II site of the plasmid pCKIZ [1]. Restriction fragments derived directly from the genomic clone were cloned into pUC119 plasmid. Using these subclones, two viral DNA fragments containing a consensus TATA box motif were isolated (Fig. 1). A fragment designated CVP1 encompassed CVMV nucleotides 7235 to 7623 and was obtained by *Alu*I enzymatic digestion. A larger fragment containing CVMV nucleotides 7160 to 7675 and designated CVP2, was isolated by PCR amplification. The two oligonucleotides used in the PCR reaction were: primer 1, 5′-ACCGGTACCAGAAGGTAATTATCCAAGATGT-3′ (CVMV sequence from 7160 to 7183 with the addition of a *Kpn*I restriction site at the 5′ end) and primer 2, 5′-CGGAATTCAAACTTACAAATTTCTCTGAAG - 3′ (CVMV sequence complementary to nucleotides 7675 to 7652 with the addition of an *Eco*RI restriction site at the 5′ end). The amplification reaction contained 25 pmol of each primer, 200 μM each dNTP, 100 ng of plasmid DNA containing the sequence to be amplified, 2.5 U *Pfu* polymerase and the appropriate buffer (Stratagene). Initial denaturation was performed at 94 °C for 5 min then the reaction mixture was denatured at 94 °C for 1 min, annealed at 60 °C for 1 min and elongated at 72 °C for 1 min for each of 15 cycles. Final extension was carried out for 5 min at 72 °C. Sequence accuracy of the amplification product was subsequently confirmed by dideoxynucleotide chain-termination sequencing (Sequenase - USB).

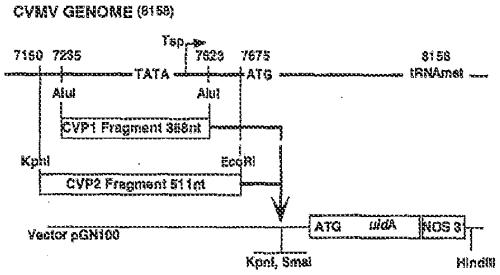


Figure 1. Construction of *uid*A fusion genes. Positions of the promoter fragments are numbered in the CVMV genomic DNA. The CVP1 fragment was isolated using *Alu*I restriction sites while the CVP2 fragment, which included an additional 75 nucleotides at the 5′ end and 52 nucleotides at the 3′ end, was obtained by PCR amplification using appropriate primers (see Materials and methods for details). The 3′ end of CVP2 is just upstream of the first ATG codon in the viral genome. The transcription start site and the position of the consensus tRNA binding site are also indicated. This chart is not drawn to scale. (Tsp, transcription start site)

Plasmid pILTAB:CVP1 (Fig. 1) was constructed by ligating the *Alu*I CVP1 fragment into SmaI site of pGN100, a pUC 119 derived plasmid containing the *uid*A-coding sequence linked to the 3′ polyadenylation signal of the nopaline synthase gene (nos 3′). The CVP2 fragment obtained from the PCR reaction was digested by *Kpn*I/*Eco*RI and subsequently blunted at the *Eco*RI 3′ end using DNA polymerase I large (Klenow) fragment. The resulting fragment was cloned in *Kpn*I/*Sma*I of pGN100 to obtain pILTAB:CVP2 plasmid (Fig. 1). The cas-

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settes containing the CVMV promoter:*uid*A fusion genes were excised by *KpnI*/*Hind*III digestion from pILTAB:CVP1 and pILTAB:CVP2 and subcloned at *KpnI*/*Hind*III sites in the pBIN19 binary vector (Clontech) used for *Agrobacterium*-mediated plant transformation. The plasmid pe35GN contains the enhanced 35S promoter [26] and the *uid*A coding sequence linked to the nos 3′ end. The plasmid pDO432 contains the luciferase coding sequence from *Photinus pyralis* under the control of the 35S promoter [40]. Plasmids pILTAB:CVP1, pILTAB:CVP1 and pe35GN used in transient assay experiments are each ca. 5.5 kb in size.

Plant transformation

Gene constructs containing pBIN19 plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation [47]. The transformed *Agrobacterium* were used to infect *Nicotiana tabacum* cv. Xanthi NN leaf discs, according to the procedure described by Horsch *et al.* [22]. Regenerated kanamycin resistant plants were transferred to soil and grown in a greenhouse. Seven independent transgenic lines containing the CVP1 construct and eight containing the CVP2 construct were produced. Greenhouse grown plants were allowed to self-fertilize and R1 seeds were collected. The R1 seeds were germinated on Murashige and Skoog (MS) medium [36] containing kanamycin 100 mg/l and the seedlings were grown in greenhouse.

Seven transgenic rice lines (*Oryza sativa* L. Taipei 309) were obtained via particle bombardment as described by Li *et al.* [31], using pILTAB:CVP2 in association with pMON410 (Monsanto Co.); the latter carries the gene for resistance to hygromycin.

Histochemical localization of β-glucuronidase activity

Histochemical analysis of GUS activity was performed essentially as described by Jefferson *et al.* [24]. Small fragments of leaf and stem from primary transformants or R1 progeny were incubated at 37 °C for 4 to 8 hs in reaction buffer containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 100 mM sodium phosphate buffer pH 7, 2 mM potassium ferrocyanide and potassium ferricyanide, and 0.1% Triton X-100. Roots and floral organs were incubated in the same medium lacking the cyanide salts and including 0.1% mercaptoethanol to reduce tissue browning. Hand-cut tissue sections were taken and cleared in 70% ethanol. Stained sections were visualized in a Zeiss microscope.

RNA preparation and primer extension analysis

Total RNA was extracted from young leaves of transgenic tobacco plants as described by Prescott and Martin [41], with minor modifications. An additional phenol/chloroform extraction was performed, followed by three successive LiCl precipitations.

Primer extension was performed with a 34 bp long oligonucleotide with the sequence 5′-CGCGATCCAG ACTGAATGCCCACAGGCCGTCGAG-3′ which is complementary to a region 34 nucleotides downstream of the ATG start codon in the *uid*A gene. The oligonucleotide (20 pmol) was 5′ end-labeled using 6 U of T4 polynucleotide kinase (USB) and 7 μCi of [γ^{32}P]ATP (3000 μCi/mmol, 10 μCi/μl). After the labeling reaction, the primer was purified using Nuctrap Push columns (Stratagene). One-tenth pmol of the labeled primer was mixed with 50 μg of total RNA from transgenic plants. The experiment was performed according to Sambrook *et al.* [44] except that annealing was carried out at 30 °C or 40 °C for 12 h, and the extension reaction was allowed to proceed for 1 h at 42 °C with 20 U of AMV reverse transcriptase (Gibco-BRL). The extension products were separated on a 7.5% polyacrylamide gel containing 7 M urea. Sequence reactions (Sequenase-USB) performed with the same labeled primer were subjected to electrophoresis in adjacent lanes on the same gel.

Protoplast isolation, treatment and culture

Tobacco protoplasts were isolated from BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow) cell suspension cultures and transfected with DNA essentially as described by Kikkawa *et al.* [27]. Cassava protoplasts were prepared from *Manihot esculenta* L. cv. TMS60444 embryogenic cell suspension cultures [48]. Fifty ml of a 10-day-old culture were collected for protoplast isolation. Prior to enzymatic digestion, the cells were resuspended in 30 ml of medium containing 0.55 M mannitol, 3.2 g/l Schenk and Hilderbrandt salts (Sigma), 1× Murashige and Skoog vitamins (Sigma), 20 mM $CaCl_2$, pH 5.8 (medium A). The cells were allowed to settle and medium A was replaced by an enzymatic solution consisting of medium A supplemented by 2% cellulase Onozuka RS and 0.1% Pectolyase Y 23. Digestion was performed in the dark for 3.5 h at 27 °C. Cells were gently agitated during the first

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hour of treatment. The incubation mixture was filtered sequentially through sieves of 100 μm and 70 μm. Protoplasts were washed three times by centrifugation at 100 × *g* for 10 min in medium A. The number of protoplasts was estimated using a hemocytometer.

The purified protoplasts were resuspended to final density of 10^6 cells/ml in electroporation buffer containing 5 mM MES, 130 mM NaCl, 10 mM $CaCl_2$, 0.45 M mannitol pH 5.8. Two hundred μl of electroporation buffer containing 30 μg of each plasmid was added to 800 μl of protoplast suspension in a 0.4 cm path-length cuvette. DNA uptake was carried out using a Gene Pulser instrument (BioRad) delivering a 300 V pulse at a capacitance of 500 μF. After electroporation, the protoplasts were incubated in ice for 30 min, after which they were resuspended at a density of 10^5 cell/ml in culture medium A supplemented with 20% sucrose and 5×10^{-5} M Pichloran. After 24 h of incubation in the dark at 27 °C, the protoplasts were collected by centrifugation (10 min at 100 × *g*) and resuspended in GUS extraction buffer [24], pH 7.7.

LUC and MUG assays

Transfected protoplasts were lysed by vortexing for 2 min in GUS extraction buffer, pH 7.7. Extracts were clarified by centrifugation (5000 × *g*, 5 min) at 4 °C in a microcentrifuge. The supernatant was recovered and used for MUG and LUC assays. GUS activity was determined using 4-methyl-umbelliferyl-β-D-glucuronide (MUG-Sigma) by the method of Jefferson [24] and quantified for 50 μl of extract as pmol methylumbelliferone (MU) per hour. LUC activity was determined on 50 μl of the same protein extract with a luminometer (Monolight 2010) using a luciferase assay (Analytical Luminescence Laboratory, San Diego, CA). Cotransfection of cells with a *uid*A gene plus a luciferase plasmid allowed us to normalize variations of GUS activity between experiments [30]. The normalized GUS data were expressed as pmol methylumbelliferone (MU) per hour per unit of light emitted.

Transgenic plant tissues were ground in GUS buffer, pH 8, and GUS activity was evaluated as described [24]. The enzyme activity (pmol/min) was referred to mg protein as determined by the dye-binding method of Bradford [9].

Particle bombardment

Leaves and stems were cut from cassava plantlets (cultivar Mcol 1505) grown *in vitro* on medium containing MS salts and vitamins, sucrose 20 g/l, $CuSO_4$ 2 μM, Phytagel 3 g/l, pH 5.7. The explants were sectioned and the tissue fragments were subsequently arranged in the center of 9 cm Petri dishes containing solidified culture medium. Micro-bombardment was performed with an helium-driven particle delivery system (PDS 1000/He-BioRad). Preparation of gold particles (average diameter 1.6 μm) and coating particles with DNA were carried out essentially as described by the instruction manual (BioRad). The target plates were placed in the gun chamber at the third level from the bottom while the assembly macrocarrier/stopping screen was placed at the fifth level. Each plate was shot twice at a pressure of 1100 PSI with ca. 1 μg of plasmid DNA. After bombardment, sterile water was added to the plates to prevent desiccation of the material. Explants were incubated 2 days in the dark at 25 °C prior to histochemical GUS assays.

Results

Isolation of the CVMV promoter

A genomic clone of cassava vein mosaic virus (CVMV) was kindly provided by Dr R. J. Shepherd (University of Kentucky) for these studies. Sequence analysis of the CVMV clone allowed us to identify in the viral genome, an AT rich region similar to that of the plant pararetroviruses TATA box regions (Fig. 2A). The putative TATA box region is located 588 nucleotides upstream of another consensus sequence that is complementary to the 3′ end of a plant $tRNA^i_{met}$; this sequence is present in all plant pararetroviruses and serves as a primer binding site to initiate the viral replication process [21]. The location of the putative TATA box sequence in CVMV is similar to the promoters that control the transcription of the genome length transcripts in CaMV [18], FMV [43] and RTBV [20, 42].

This information allowed us to predict the location of a putative promoter in the CVMV genome. Two DNA fragments were subsequently isolated and tested for promoter expression: CVP1 comprising 387 nucleotides from position −368 to +20 (+1 corresponds to the transcription start site reported in this paper), and CVP2, a fragment of 514 nucleotides

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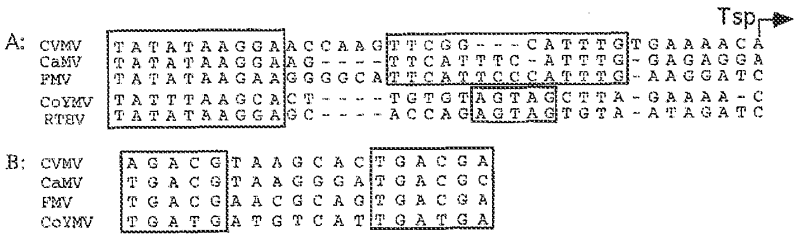


Figure 2. Conserved nucleotide motifs in plant pararetrovirus promoters. A. Comparison of nucleotide sequences from the TATA box to the transcription start site of promoters from plant pararetroviruses [2, 19, 33, 45]. Regions of high homology are framed. Tsp indicates the transcription start point of these promoters. B. Sequence alignment of different as1 like elements found in the CaMV, FMV, CoYMV and CVMV promoter. CaMV and FMV have been characterized as caulimoviruses [18, 43] whereas CoYMV and RTBV are badnaviruses [33, 42].

from position −443 to +72 (Fig. 1). Both fragments were cloned into a plasmid that contains the *uid*A-coding sequence for analysis of promoter activity. The nucleotide sequences of the promoter fragments are presented in Fig. 3 and were confirmed by the results reported by Calvert *et al.* [10]. A comparison with other pararetrovirus promoter sequences, namely the 35S CaMV[18], the 34S FMV [43], RTBV [42], and CoYMV [33] promoters revealed the presence of a conserved TATA box as mentioned previously, and a 17 nucleotide motif AGACGTAAGCACTGACG (position −203 to −219) that has strong homology with the transcriptional enhancer as1 found in the 35S CaMV, FMV, and CoYMV promoters (Fig. 2B). We also identified a 22 nucleotide sequence CTTATCACAAAGGAATCTTATC (position −90 to −111) that is present in the CoYMV promoter but not in RTBV or caulimovirus promoters. This limited homology suggested that we had isolated a distinct plant pararetrovirus promoter.

Determination of the transcription start site

The transcription start site of the CVMV promoter was determined by primer extension analysis using total RNA recovered from transgenic tobacco plants which harbored the CVP1: *uid*A fusion gene (Fig. 1). A single major extension product was detected and mapped to an adenine residue (nt 7604) located 35 nucleotides downstream of the putative TATA box (Fig. 4). By comparison with the location of the start of transcription reported for other plant pararetrovirus promoters [2, 19, 33, 45] (Fig. 2A), the transcription start site reported here likely represents the $5'$ end of the CVMV transcript. By comparing sequence of the CVMV promoter from the TATA box to the start of transcription with those of caulimovirus promoters FMV 34S and CaMV 35S, we concluded that these three promoters are, in this region, closely related to each other (Fig. 2A). In contrast there is less homology with the promoters from badnaviruses (CoYMV and RTBV).

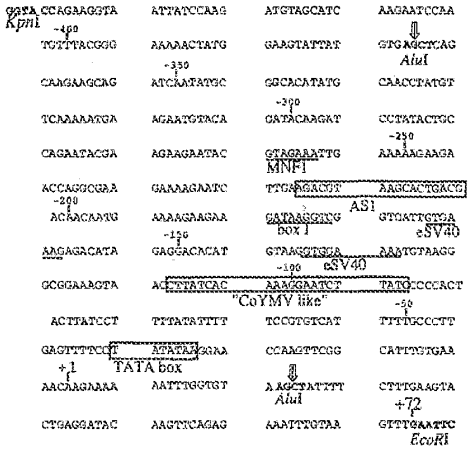


Figure 3. Nucleotide sequence of CVP1 and CVP2 promoter fragments. The transcription start site is designated +1. Consensus TATA box, as 1-like sequence [29] and the region homologous with the CoYMV promoter [35] are boxed. *Alu*I sites (⇩)indicate the $5'$ and $3'$ ends of the CVP1 promoter. Sequence motifs similar to previously characterized *cis* elements in plant promoters are underlined (motifs with similarities with the box I element [13] of the *rbcS* promoter, MNF1-binding site [50], SV40 core enhancer [38] are indicated).

Expression of the CVMV promoter in protoplasts

Promoter fragments CVP1 and CVP2 were tested in transient assay experiments using tobacco and cassava protoplasts obtained from cell suspension cultures. We

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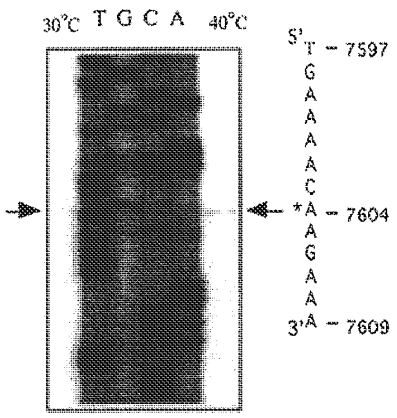


Figure 4. Determination of the transcription start site of the CVMV promoter. Primer extension reactions were carried out as described in Materials and methods. The products of the extension reactions, obtained with two annealing temperatures (30 °C and 40 °C) and reference sequencing reactions of CVP1-*uid*A gene construct (lanes A, C, G and T) performed with the same labeled primer, were subjected to electrophoresis in a 7 M urea, 7.5% polyacrylamide gel. The plus strand DNA sequence (complementary to the sequence read on the gel) is shown and the transcription start site (A*) is indicated by an arrow. Numbers correspond to the nucleotide sequence numbers of the CVMV genome [10].

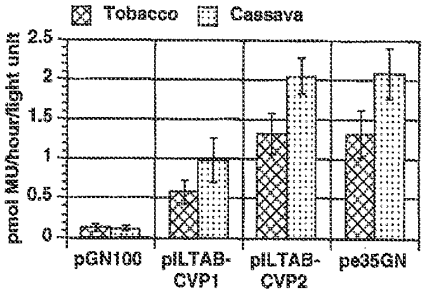


Figure 5. Expression of the CVMV promoters in tobacco and cassava protoplasts. Chimeric *uid*A gene constructs were co-introduced with a plasmid to express luciferase [40] into protoplasts of cassava and tobacco via electroporation. Promoter activity is expressed as a ratio between GUS activity and LUC activity of the same protein extract. As a consequence, GUS activity is measured as pmol 4-methylumbelliferyl-β-D- glucuronide (MUG) per hour per unit of light emitted. Bars represent the average of four independent experiments ± standard errors. Each experiment was carried out with a different protoplast preparation. pe35GN is a construct where the enhanced 35S promoter [26] controls the transcription of the *uid*A gene. pGN100 contains a promoter-less *uid*A gene.

used in this experiment, the plasmids pILTAB:CVP1 and pILTAB:CVP2. The plasmid pe35GN, containing the *uid*A sequence under the control of the enhanced 35S promoter (e35S) [26], served as a positive control. Each plasmid was cointroduced into protoplasts with a plasmid containing a luciferase gene under the control of the CaMV 35S promoter [40]. The GUS/LUC ratio was determined after each transfection experiment. Four independent transfection experiments, summarized in Fig. 5, were carried out and gave similar results. In tobacco protoplasts the GUS/LUC ratio for the CVP1 promoter was 0.58, or about 50% of the level of expression determined by the e35S promoter (1.32). However, when the CVP2 fragment was used, the ratio was 1.3, or two-fold more active than CVP1. The difference between the two fragments indicated that CVP1 lacks one or more important element(s) for high level expression. CVP2 and e35S promoters yielded similar GUS activity indicating that the CVMV promoter is a strong promoter in tobacco protoplasts. Similar studies in cassava protoplasts gave results comparable to those in tobacco showing that the CVMV promoter is also very effective in these cells.

Expression of the CVMV promoter in transgenic tobacco and rice plants

Seven transformed tobacco lines containing CVP1 promoter-*uid*A fusion gene and eight containing CVP2 promoter were obtained as described in Materials and methods. Presence of the full length gene cassette was confirmed by PCR analysis (data not shown) of primary transformants (plants regenerated from transgenic calli). A detailed histochemical analysis of GUS accumulation was carried out using hand-cut fresh tissue sections of various organs from primary transformed plants and their R1 progeny. All transformed tobacco plants containing either the CVP1 or CVP2 fragment had essentially the same gene expression pattern while intensity of staining varied among transformants. In leaves, strong GUS activity was observed in phloem tissues in the midrib and in the lateral secondary veins (Fig. 6A, B). Parenchyma cells adjacent to xylary elements also developed a blue staining pattern while the parenchyma cells of the midrib did not contain detectable GUS activity (Fig. 6A, B) except for the chlorenchyma cells just below the epidermis (Fig. 6C). The cells of the palisade layer and the spongy mesophyll in the leaf lamina exhibited a very intense staining (Fig. 6A, C), while in the epidermis, guard cells and trichomes, especially the glandular tip cell, developed an intense staining. Non-specialized

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epidermal cells accumulated little or no stain. Cross sections of stems showed strong staining of the phloem cells, including internal phloem bundles located in the central pith tissue and phloem cells located external to the xylem (Fig. 6D). Weaker expression was also visible in the xylem parenchyma cells. GUS staining was not detectable in pith cells or in cortical parenchyma cells of the stem (Fig. 6D). Root tissues incubated with X-Gluc revealed a blue stained vascular cylinder (Fig. 6E); cross-sections were not taken due to the fragile nature of the tissue. The root tips stained the most intensely of any region in the root (Fig. 6E). In the flowers, the basal part of the ovary exhibited an intense blue staining. The vascular elements of floral tissue displayed a strong staining in the stamen, the style and the placenta inside the ovary (Fig. 6F), as well as in the sepals and petals. Pollen also exhibited a blue color (not shown). R1 seedlings developed the same general pattern of staining as did the R0 parental transformant except that GUS activity in the mesophyll of cotyledons appear weaker than in mature leaves.

Histochemical analysis to detect GUS activity was performed in a similar manner on 7 independently transformed rice lines that harbored the CVP2: *uid*A gene. The general expression pattern of the CVP2 promoter-*uid*A gene was quite similar in rice and tobacco, despite the differences in anatomy of these plants. Transverse sections of leaves incubated with X-Gluc substrate resulted in strong staining in the vascular bundles and in the mesophyll cells (Fig. 6G). The small phloem parenchyma cells and the xylem parenchyma cells exhibited an intense staining while the metaxylem tracheary elements and the larger sieve elements appeared to be free of any blue precipitate. Bundle sheath cells, bulliform cells and sclerenchyma fibers also showed no staining. Guard cells and leaf hair cells were stained in the leaf epidermis. The pattern of GUS activity revealed in cross-sections of the leaf sheath tissue (Fig. 6H) was similar with that observed in leaves. As observed in tobacco plants, GUS activity was not detectable in parenchyma cells (Fig. 6H). Roots were stained only in the vascular cylinder and in the tip. Rice floral tissue had essentially the same pattern of GUS activity as the tobacco flowers (Fig. 6I).

GUS activity in extracts prepared from different organs was determined among tobacco and rice transformed plants using the 4-methylumbelliferyl-β-D-glucuronide (MUG) fluorescence assay [24]. The organs tested included young leaves, mature leaves, stem and root (Fig. 7). This experiment showed that the CVMV promoter (CVP1 and CVP2 constructs) is expressed in all organs of transformed rice and tobacco. These results supported the GUS histochemical analysis of transgenic plant presented previously, and confirmed the constitutive property of the cassava vein mosaic virus promoter. We observed that the CVMV promoter was more active in leaves than in others organs while the lowest level of expression was in roots. The GUS expression levels, measured for the rice leaf sheath, were very close to those measured in leaves. This could be due to the presence of photosynthetic mesophyll tissue at the periphery of rice leaf sheath (see Fig. 6H for anatomic detail). In tobacco, GUS expression in the plants harboring the CVP2 promoter construct appeared slightly stronger than in plants containing the CVP1 promoter. However, this result has to be confirmed by more extended analysis, due to the relative low number of lines tested and to the variation observed in the transgene expression between lines. The latter could be due to a putative position effect or/and to the different copy number of the inserted gene.

Activity of the CVMV promoter in cassava plant explants

The promoter activity of the CVP2 fragment (Fig. 1) was tested in cassava plants by micro-particle bombardment on stem and leaf explants from material grown *in vitro*. The plasmid pILTAB:CVP2 and plasmid pe35GN (as positive control) were used in this study. Approximately the same number of intensely blue-stained foci showing GUS expression (Fig. 6J) were found using plasmids containing either promoter. Blue-stained cells were found in epidermal cells, guard cells, mesophyl cells and along the veins of leaflets. These experiments provided evidence of promoter activity for CVP2 fragments in different cell types of cassava.

Discussion

We have isolated a promoter from the viral genome of the newly characterized cassava vein mosaic virus [10]. The transcription start site of the promoter was determined using RNA isolated from transgenic plants that contain the pCVMV-*uid*A gene. Our results indicate that the CVMV promoter is strong in tobacco and cassava protoplasts and its activity is similar to that obtained with the e35S promoter. Of the two promoter fragments tested in protoplasts, the shorter frag-

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