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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		
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☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

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Inventor 1					Remove
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number		DAS-P0129-04-US-E	
		Application Number			
Title of Invention		NOVEL HERBICIDE RESISTANCE GENES			
City	Zionsville	State/Province	IN	Country of Residence i	US
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number		DAS-P0129-04-US-E	
		Application Number			
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES				
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All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.					Add

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Email Address	intear@faegrebd.com	Add Email	Remove Email

Application Information:

Title of the Invention	NOVEL HERBICIDE RESISTANCE GENES		
Attorney Docket Number	DAS-P0129-04-US-E	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	3	Suggested Figure for Publication (if any)	

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For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

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☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)
Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

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Prior Application Status	Pending		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
	Continuation of	13647081	2012-10-08		
Prior Application Status	Patented		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12091896	a 371 of international	PCTUS2006042133	2006-10-27	8283522	2012-10-09
Prior Application Status	Expired		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
PCTUS2006042133	Claims benefit of provisional	60731044	2005-10-28		
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		
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Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

<p>☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.</p> <p>NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.</p>
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Applicant 1[Remove](#)

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Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E	
		Application Number		
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES			

Prefix	Given Name	Middle Name	Family Name	Suffix

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Signature	/yonghao hou/		Date (YYYY-MM-DD)	2014-09-19	
First Name	Yonghao	Last Name	HOU	Registration Number	61391

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NOVEL HERBICIDE RESISTANCE GENESCross References

This application is a continuation of U.S. Patent Application Serial No. 13/647,081, filed on October 8, 2012, which is a continuation of U.S. Patent 8,283,522 with Serial No. 12/091,896, filed on November 3, 2008, which claims the benefit of PCT International Application Serial No. PCT/US2006/042133, filed on October 27, 2006, which claims the benefit of U.S. Provisional Application Serial No. 60/731,044, filed on October 28, 2005, the disclosures each of which are expressly incorporated herein by reference.

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; Sfiligoi, 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 (Agrilience, 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 (SEQ ID NO:2), TfdA (SEQ ID NO:18), AAD-2 (SEQ ID NO:19), AAD-1 (SEQ ID NO:20), and TauD (SEQ ID NO:21).

[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 “NcoI of Brady” primer.

[0037] **SEQ ID NO:15** shows the sequence of the “SacI of Brady” primer.

[0038] **SEQ ID NO:16** provides the sequence of forward primer brjap 5' (speI).

[0039] **SEQ ID NO:17** provides the sequence of reverse primer brjap 3' (xhoI).

[0040] **SEQ ID NO:18** provides the sequence of tfdA.

[0041] **SEQ ID NO:19** provides the sequence of AAD-2.

[0042] **SEQ ID NO:20** provides the sequence of AAD-1.

[0043] **SEQ ID NO:21** provides the sequence of tauD.

Detailed Description of the Invention

[0044] 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 O₂ 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	
MCPA	94-74-6	25 – 4000	125 - 1550	
Triclopyr	55335-06-3	50 – 2000	70 – 840	
Fluroxypyr	69377-81-7	25 – 2000	35 – 560	

[0045] 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.

[0046] 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).

[0047] 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.

[0048] 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.

[0049] 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.

[0050] 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, pyrimidinylthiobenzoates, and

other chemistries = *AHAS*, *CsrI*, *SurA*, *et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxyphenyl-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-I*), 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.

[0051] 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).

[0052] 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.

[0053] 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)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R” 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.

[0054] 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.

[0055] 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.

[0056] 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.

[0057] 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.

[0058] 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.

[0059] 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.

[0060] 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*.

[0061] 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).

[0062] 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).

[0063] 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.

[0064] 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 (*CryIF*, *CryIAb*, *Cry 34/45*, other Bt. Proteins, or insecticidal proteins of a non-*Bacillus* 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.

[0065] 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.

[0066] 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.

[0067] 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.

[0068] 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.

[0069] 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.

[0070] 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.

[0071] 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.

[0072] 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.

[0073] 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.

[0074] 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.

[0075] 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.

[0076] 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.

[0077] 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.

[0078] 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.

[0079] 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.

[0080] 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.

[0081] 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.

[0082] 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.

[0083] 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.

[0084] 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.

[0085] 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.

[0086] 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.*).

[0087] 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.

[0088] 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.

[0089] 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.

[0090] 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 ³²P-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 (T_m) 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):

$$T_m = 81.5^{\circ} \text{ C} + 16.6 \text{ Log}[\text{Na}^+] + 0.41(\% \text{G+C}) - 0.61(\% \text{formamide}) - 600/\text{length of duplex in base pairs.}$$

[0091] 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 $T_m - 20^\circ \text{C}$ for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

[0092] For oligonucleotide probes, hybridization can be carried out overnight at $10-20^\circ \text{C}$ below the melting temperature (T_m) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T_m for oligonucleotide probes can be determined by the following formula:

$$T_m (^\circ\text{C}) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(Suggs *et al.*, 1981).

[0093] 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).

[0094] 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 .

[0095] 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.

[0096] 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.

[0097] 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.

[0098] 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.

[0099] 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; *Rhodoferrax*) and AJ6288601.1 (44903451; *Sphingomonas*). *Rhodoferrax* is very similar to *Delftia* but *Sphingomonas* is an entirely different Class phylogenetically.

[00100] 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.

[00101] 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.

[00102] “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.

[00103] Specific changes to the “active site” of the enzyme can be made to affect the inherent functionality 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 *Escherichia 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.

[00104] 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.

[00105] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal31* 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.

[00106] 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.

[00107] 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.

[00108] 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.

[00109] 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.

[00110] 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

[00111] 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.

[00112] 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.”

[00113] 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.”

[00114] 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.

[00115] 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., alfalfa 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.

[00116] 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.

[00117] 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.

[00118] 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.

[00119] 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.

[00120] 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.

[00121] 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).

[00122] 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.

[00123] 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.

[00124] 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.

[00125] 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.

[00126] 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.

[00127] 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.

[00128] 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.

[00129] 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.

[00130] 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.

[00131] 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, sulfonyleureas and triazolopyrimidine herbicides), ACC-ase inhibitors (e.g., aryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

[00132] In addition to a selectable marker, it may be desirable 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.

[00133] 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.

[00134] 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.

[00135] 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.

[00136] 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 known in the art, and can foreseeably be optimized and further developed, for increasing expression of a given gene.

[00137] 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.

[00138] 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.

[00139] Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

[00140] 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.”; Cramer 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; Cramer, 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.

[00141] 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.

[00142] 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.

[00143] 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).

[00144] 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.

[00145] Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve desired traits.

[00146] 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.)

[00147] 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.

[00148] 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.

[00149] 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.

[00150] 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, amiprofos-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-dinitroanilines (trifluralin and pendimethalin), phosphamidates (amiprofos-methyl, thiocarbamates (pyributicarb), and ureas (chlortoluron)).

[00151] 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).

[00152] Auxin transport inhibitors (e.g. diflufenzopyr) can also be used to increase herbicide activity with 2,4-D.

[00153] Unless specifically indicated or implied, the terms “a”, “an”, and “the” signify “at least one” as used herein.

[00154] 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.

[00155] 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*

[00156] 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 $\leq 50\%$ 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.

[00157] 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

[00158] 2.1 – Background.

[00159] 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 redesign 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.

[00160] 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.

[00161] **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 MacVector™ program (Accelrys, 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

[00162] 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.

[00163] 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

[00164]

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.

[00165] 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.

[00166] 2.2 – *AAD-12* Plant rebuild analysis.

[00167] 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		TTC	7	5	5
ASN (N)	AAC	3	2	2	PHE (F)	TTT	1	3	3
	AAT	1	2	2		CCA	0	5	6
ASP (D)	GAC	15	9	9	PRO (P)	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		AGC	5	4	3
END	TAA	1	0	1	SER (S)	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		TAC	4	3	3
HIS (H)	CAC	8	9	9	TYR (Y)	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

[00168]

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.

[00169] 2.3 Rebuild for *E. coli* Expression

[00170] 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 Umberger, 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.

[00171] 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 #	<i>E. coli</i> Opt v2 #	Theor. Class II #	Amino Acid	Codon	Native #	<i>E. coli</i> 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	LYS (K)	TTG	0	0	0
	CGA	0	0	0		AAA	1	4	5
	CGC	15	6	6	AAG	AAG	5	2	1
	CGG	3	0	0		ATG	10	10	10
CGT	CGT	0	12	12	PHE (F)	TTC	7	6	6
	AAC	3	4	4		TTT	1	2	2
ASN (N)	AAT	1	0	0	PRO (P)	CCA	0	3	2
	GAC	15	10	9		CCC	9	0	0
ASP (D)	GAT	2	7	8		CCG	5	11	12
	TGC	3	2	2		CCT	0	0	0
CYS (C)	TGT	0	1	1	SER (S)	AGC	5	4	4
	TAA	1	1	1		AGT	0	0	0
END	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		TAC	4	3	3
HIS (H)	CAC	8	11	11	TYR (Y)	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

[00172] 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.

[00173] 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.

[00174] 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:

$$\text{Weighted \% of C1} = 1/(\%C1 + \%C2 + \%C3 + \text{etc.}) \times \%C1 \times 100$$

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
					TCG	DNU	6.1
GLN (Q)	CAA	55.5	55.5		TCT	25.8	24.2
	CAG	44.5	44.5	THR (T)	ACA	32.4	29.7
GLU (E)	GAA	50.5	50.5		ACC	30.2	27.7
	GAG	49.5	49.5		ACG	DNU	8.3
GLY (G)	GGA	31.9	31.9		ACT	37.4	34.3
	GGC	19.3	19.3	TRP (W)	TGG	100.0	100
	GGG	18.4	18.4		TAC	48.2	48.2
	GGT	30.4	30.4	TYR (Y)	TAT	51.8	51.8
HIS (H)	CAC	44.8	44.8	VAL (V)	GTA	11.5	11.5
	CAT	55.2	55.2		GTC	17.8	17.8
ILE (I)	ATA	23.4	23.4		GTG	32.0	32.0
	ATC	29.9	29.9		GTT	38.7	38.7
	ATT	46.7	46.7				

*DNU = Do Not Use

[00175] 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.

[00176] 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

[00177] 3.1 Construction of E.coli, pET expression vector.

[00178] 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.

[00179] 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.

[00180] 3.2- Construction of *Pseudomonas* expression vector

[00181] 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.

[00182] 3.3 – Completion of binary vectors.

[00183] 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).

[00184] 3.4 - Cloning of additional transformation constructs.

[00185] 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* CsVMV = Cassava Vein Mosaic Virus Promoter
 ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
 T = Tobacco AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
 HptII = hygromycin phosphotransferase
 S = Soybean Atubi3= *Arabidopsis thaliana* Ubiquitin 3 Promoter
 Ct = Cotton AtAct2= *Arabidopsis thaliana* Actin 2 Promoter
 R = Rice RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)
 Cn = Corn Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and
 the *Nicotiana tabacum* Osmotin 3' Untranslated Region
 Ca = Canola

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

[00186] 4.1- *Pseudomonas fluorescens* fermentation

[00187] 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; K₂HPO₄, 5 g/L; (NH₄)₂PO₄, 7.5 g/L; (NH₄)₂SO₄; MgSO₄·7H₂O, 1 g/L; KCl, 0.5 g/L; CaCl₂·2H₂O, 0.5 g/L; NaCitrate-2H₂O, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: FeCl₃·6H₂O, 5.4 g/L; MnCl₂·4H₂O, 1 g/L; ZnSO₄·7H₂O, 1.45 g/L; CuSO₄·5H₂O, 0.25 g/L; H₃BO₃, 0.1 g/L; (NH₄)₆MO₇O₂₄, 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.

[00188] 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 OD₅₇₅. 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.

[00189] 4.2- Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

[00190] 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 OD₂₈₀ 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 OD₂₈₀ 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.

[00191] 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.

[00192] 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.

[00193] 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

[00194] 5.1 – Assay via colorimetric phenol detection.

[00195] 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 $\text{NH}_4(\text{FeSO}_4)_2$, 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.

[00196] 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.

[00197] 5.2 – Assay via detection of chloropyridinol

[00198] 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 $\sim 8,400 \text{ M}^{-1}.\text{cm}^{-1}$). 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 μ l final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9 μ M chloropyridinol.

[00199] 5.3 – Colorimetric assay using 2-(2-chloro,4-nitrophenoxy)propionate

[00200] 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 μ M NH_4FeSO_4 , 200 μ M 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 μ l final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1 μ M 2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be $K_m = 31 \pm 5.5 \mu\text{M}$ and $k_{\text{cat}} = 16.2 \pm 0.79 \text{ min}^{-1}$.

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

[00201] 6.1 – AAD-12 (v2) activity on (R,S)- dichlorprop, (R)-dichlorprop, (S)-dichlorprop and 2,4-D

[00202] Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4 μ g 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 μ g 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

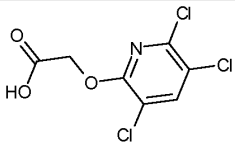
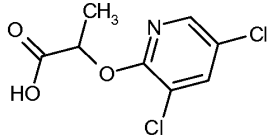
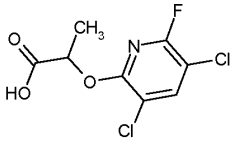
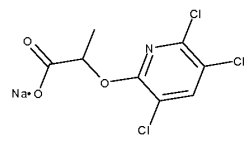
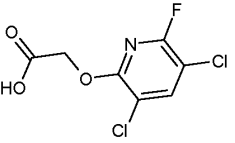
determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

[00203] 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.

[00204] 6.2 – AAD-12 (v2) activity on pyridyloxyalkanoates

[00205] 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

[00206]

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

[00207]

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 ($\pm\text{SE}$)	k_{cat} , min^{-1} ($\pm\text{SE}$)	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)	$\Delta\text{A}_{325\text{ nm}}$	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

[00208] 7.1 – *Arabidopsis thaliana* growth conditions.

[00209] 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).

[00210] 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.

[00211] 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 $\mu\text{mol}/\text{m}^2\text{sec}$ 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.

[00212] 7.2 – *Agrobacterium* transformation.

[00213] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5 α 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.

[00214] 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.

[00215] 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.

[00216] 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.

[00217] 7.3 – *Arabidopsis* transformation.

[00218] *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.

[00219] 7.4 – Selection of transformed plants.

[00220] Freshly harvested T₁ seed [*AAD-12 (v1)* gene] was allowed to dry for 7 days at room temperature. T₁ 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 T₁ 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.

[00221] 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).

[00222] Seven days after planting (DAP) and again 11 DAP, T₁ 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 µE/m²s⁻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.

[00223] T₁ 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.

[00224] 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.

[00225] Some T₁ 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 T₁ plants with use of a track sprayer in a 187 L/ha spray volume. T₁ plants that exhibited tolerance to 2,4-D DMA were further accessed in the T₂ generation.

[00226] 7.5 – Results of selection of transformed plants.

[00227] The first *Arabidopsis* transformations were conducted using *AAD-12 (v1)* (plant optimized gene). T₁ transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T₁ 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. T₁ 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* T₁ 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 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. The *AAD-12 (v1)* gene imparted herbicide resistance to individual T₁ *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 T₁ *Arabidopsis* response to a range of 2,4-D rates applied postemergence, compared to an *AAD-1* v3 (T₄) homozygous resistant population, or a *Pat-Cry1F* transformed, auxin-sensitive control.

AAD-12 (v1) gene T₁ transformants	% Injury				% Injury	
	Averages	<20%	20-40%		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%			
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 T₄ plants	% Injury				% Injury Ave	Std Dev
	Averages	<20%	20-40%			
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

[00228]

Table 12 shows a similarly conducted dose response of T₁ *Arabidopsis* to the phenoxypyropionic 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. T₁ *Arabidopsis* response to a range of R-dichlorprop rates applied postemergence.

AAD-12 v1 gene	% Injury				% Injury	
	Averages	<20%	20-40%		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	
T₄ 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

[00229] 7.6 – AAD-12 (v1) as a selectable marker.

[00230] 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 T₄ generation *Arabidopsis* seed (homozygous for *AAD-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 followed 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.

[00231] Treatments were applied with a DeVilbiss spray tip as previously described. 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*.

[00232] 7.7 – Heritability.

[00233] A variety of T₁ events were self-pollinated to produce T₂ seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T₂ siblings. Each individual T₂ 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 T₁ families (T₂ 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 ($P > 0.05$).

[00234] Seed were collected from 12 to 20 T₂ individuals (T₃ seed). Twenty-five T₃ siblings from each of eight randomly-selected T₂ families were progeny tested as previously described. Approximately one-third of the T₂ 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.

[00235] 7.8 – Additional foliar applications herbicide resistance in AAD-12 *Arabidopsis*.

[00236] 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. T₂ 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.

[00237] 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 controls (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 <i>Arabidopsis</i> plant response to various foliar-applied auxinic herbicides.		
Pyridyloxyacetic auxins		
Herbicide Treatment	Ave % Injury 14DAT	
	Segregating T ₂ <i>AAD-12 (v1)</i> 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 T₂ 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%.

[00238] 7.9 – Molecular analysis of AAD-12 (v1) Arabidopsis.

[00239] 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.

[00240] 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.

[00241] 7.10 – Arabidopsis transformed with molecular stack of AAD-12 (v1) and a glyphosate resistance gene.

[00242] T₁ *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (*AAD-12 (v1)* + *EPSPS*) which encodes a putative glyphosate resistance trait. T₁ transformants were selected using *AAD-12 (v1)* as the selectable marker as described in example 7.6. T₁ 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* T₄ 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. [00243] 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 differencing 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. T₁ *Arabidopsis* response to a range of glyphosate rates applied postemergence (14 DAT).

AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)	% Injury			% Injury	
	<20%	20-40%	>40%	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	% Injury			% Injury	
Averages	<20%	20-40%	>40%	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)	% Injury			% Injury	
Averages	<20%	20-40%	>40%	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		% Injury			
	<20%	20-40%				
Untreated control	5	0	0		Ave	Std Dev
26.25 g ae/ha glyphosate	0	0	5		0	0
105 g ae/ha glyphosate	0	0	5		66	8
420 g ae/ha glyphosate	0	0	5		100	0
1680 g ae/ha glyphosate	0	0	5		100	0

[00244] 7.11 – AAD-12 Arabidopsis genetically stacked with AAD-1 to give wider spectrum of herbicide tolerance.

[00245] AAD-12 (v1) (pDAB724) and AAD-1 (v3) (pDAB721) plants were reciprocally crossed and F₁ seed was collected. Eight F₁ seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F₁ 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.

[00246] One hundred F₂ seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F₂ 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-dichlorprop 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 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).

[00247] 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 novel spectrums of herbicide resistance in transgenic plants of the subject invention.

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

[00248] 8.1 – Cloning of *AAD-12 (v1)*.

[00249] 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 DH5 α 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.

[00250] 8.2 – Callus/suspension initiation.

[00251] To obtain immature embryos for callus culture initiation, F₁ 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.

[00252] 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 AgNO₃, 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.

[00253] 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.

[00254] 8.3 – Cryopreservation and thawing of suspensions.

[00255] 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.

[00256] 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.

[00257] 8.4 – Stable Transformation

[00258] 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 volume 8.1 ml of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, sterile silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00259] 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 then 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.

[00260] 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.

[00261] 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.

[00262] 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.

[00263] 8.4.1- Protocol for Isolate recovery and regeneration.

[00264] 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.

[00265] 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 µEm⁻²s⁻¹) for one week, then higher light (40 µEm⁻²s⁻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.

[00266] 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 T₁ 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. T₀ plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (*AHAS* gene) indicated insertion complexity ranging from 1 to >10 copies. Thirteen events contained the complete coding region for *AAD-12*; however, further analysis 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.

[00267] 8.5– Molecular Analysis: Maize Materials and Methods.

[00268] 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.

[00269] 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 MgCl₂ (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.

[00270] 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.

[00271] 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.

[00272] 8.5.4 – Southern blot analysis.

[00273] 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 .

[00274] 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.

[00275] 8.6 – Postemergence herbicide tolerance in AAD-12 transformed T₀ Corn.

[00276] Four T₀ 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.

[00277] 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 T₀ 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 <i>AAD-12</i> .						
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

[00278] **8.7 – Verification of high 2,4-D tolerance in T_1 corn.**

[00279] 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 <i>AAD-12 (v1)</i> 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	<i>AAD-12 (v1)</i> pDAB4101(0)003.003	<i>AAD-12 (v1)</i> pDAB4101(0)001.001	<i>AAD-12 (v1)</i> 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

[00280] 8.8 – *AAD-12 (v1)* heritability in corn.

[00281] A progeny test was also conducted on seven *AAD-12 (v1)* T₁ 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 $\geq$ 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.

[00282] 8.9 – Stacking of *AAD-12 (v1)* to increase herbicide spectrum

[00283] *AAD-12 (v1)* (pDAB4101) and elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F₁ seed was collected. The seed from two F₁ 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 F₁ 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 <i>AAD-12 (v1)</i> and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).				
Herbicide	Untransformed Control	2P782 (Roundup Ready Control)	<i>AAD-12 (v1)</i> pDAB4101(0) 003.R003.AF	<i>AAD-12 (v1)</i> pDAB4101(0) 005.R001.AF
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

[00284] 8.10- Field tolerance of pDAB4101 transformed corn plants to 2,4-D, triclopyr and fluroxypyr herbicides.

[00285] 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) control 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 F₂ 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.

[00286] *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 normal 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.

[00287] 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.

[00288] 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.					
Treatment	Rate (g ae/ha)	%Visual Injury 14DAT			
		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.

Treatment	Brace Root Injury Rating (0-10 scale) 28DAT			
	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

[00289]

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.

[00290]

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

[00291]

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 H₂SO₄. 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).

[00292] For comparison, each sample was normalized with its fresh weight and percent expression was calculated.

[00293] 9.3 – Western blotting analysis.

[00294] 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

[00295] 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 sterily for use in the transformation protocol. Pieces of approximately one square centimeter were sterily 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.

[00296] 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 $\mu\text{E}/\text{m}^2\text{s}^1$).

[00297] Prior to propagation, T₀ 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 .

[00298] 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 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 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.

[00299] 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.

[00300] 10.1 Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T₀ tobacco

[00301] T₀ 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 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 20. Tobacco T0 events transformed with pDAS1580 (*AAD-12* (*v1*) + *PAT*)

#	Plant ID	Copy #	PTU PCR	Full PTU and	Full PTU and	Relative Herbicide Tolerance [@]
Tube		<i>PAT</i>	<i>AAD-12</i>	Under 2	1 copy	
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 ac/ha fluroxypyr where tolerance was variable across events.

[00302]

10.2 Verification of high 2,4-D tolerance in T₁ tobacco.

[00303]

Two to four T₀ 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 T₁ seed. The T₁ 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 T₀ generation. Improved consistency of response is anticipated in T₁ 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 T₁ generation is segregating, so some variable response is expected due to difference in zygosity.

[00304] 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 <i>AAD-12 (v1)</i> T ₁ tobacco plants' response to various phenoxy and pyridyloxy auxin herbicides.			
Herbicide	KY160 – Wildtype	1580(1)-004 (high tolerance in T₀ generation)	1580(1)-018 (high tolerance in T₀ 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

[00305] 10.3 *AAD-12* (*v1*) heritability in tobacco

[00306] 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 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.

[00307] 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.

[00308] 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.

[00309] *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-*

I2 (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-I2 (v1)* lines demonstrated increased tolerance with an average of 11% injury at 14 DAT.

[00310] These results indicate that *AAD-I2 (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. <i>AAD-I2 (v1)</i> tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.					
<u>Herbicide Treatment</u>		<u>Average % Injury across locations at 14 DAT</u>			
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

[00311] 10.5 *AAD-I2 (v1)* protection against elevated 2,4-D rates

[00312] Results showing *AAD-I2 (v1)* protection against elevated rates of 2,4-D DMA in the greenhouse are shown in Table 23. T₁ *AAD-I2 (v1)* plants from an event segregating 3R:1S when selected with 560 g ai/ha Liberty using the same protocol as previously described. T₁ *AAD-I (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.

[00313] *AAD-I2 (v1)* and *AAD-I (v3)* both effectively protected tobacco against 2,4-D injury at doses up to 4X commercial use rates. *AAD-I2 (v1)*, however, clearly demonstrated a marked advantage over *AAD-I (v3)* by protecting up to 64X the standard field rates.

Table 23. Results demonstrating protection provided by <i>AAD-12 (v1)</i> and <i>AAD-1 (v3)</i> against elevated rates of 2,4-D.			
	KY160 control	<i>AAD-1(v3)</i>	<i>AAD-12 (v1)</i>
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

[00314] 10.6 Stacking of *AAD-12* to increase herbicide spectrum

[00315] Homozygous *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) plants (*see* PCT/US2005/014737 for the latter) were both reciprocally crossed and F₁ seed was collected. The F₁ seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regime 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 T₂ 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.

[00316] 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 (phenoxyacetic acids + phenoxypropionic acids vs phenoxyacetic 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 stacking a second tolerance gene for the same herbicide. Such can be done using the same gene with the same or different promoters; however, as observed here, stacking and tracking two complementary traits can be facilitated by the distinguishing cross protection to phenoxypropionic acids [from *AAD-1 (v3)*] or pyridyloxyacetic acids [*AAD-12 (v1)*]

Table 24. Comparison of auxinic herbicide cross tolerance of *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) T₂ plants compared to AAD-12 x AAD-1 F₁ cross and to wildtype.

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

Example 11 - Soybean Transformation

[00317] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgett *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 (Hinchey *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00318] 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)*.

[00319] 11.1 Gateway cloning of binary constructs

[00320] 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).

[00321] 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.

[00322] 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.

[00323] 11.2 Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00324] 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 (Hinchey *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).

[00325] 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 produce AAD-12 (v1) transgenic plants.

[00326] 11.2.2– *Agrobacterium* preparation and inoculation

[00327] *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.

[00328] *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 Phytigel. 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.

[00329] 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 Conviron. 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.

[00330] 11.2.3 - Assay of putatively transformed plantlets, and analyses established T₀ plants in the greenhouse.

[00331] 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.

[00332] 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 T₀ 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 trifoliolate. 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. T₀ plants will be allowed to self fertilize in the greenhouse to give rise to T₁ seed. T₁ plants (and to the extent enough T₀ 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 T₀ 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. T₁ plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T₀ and T₁ 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 T₁ 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 T₀ 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 T₁ and T₂ progeny segregation with respect to herbicide tolerance as described in previous examples.

[00333] A subset of the initial transformants were assessed in the T₀ 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 as wildtype plants to leaf paint applications of 2,4-D

[00334] 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. Wildtype 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				Leaf flip assay										
(pDAB#)	Gene	Promoter	Event	NODE	2,4-D @ (18 HAT)									
				LEAF	Node		Spray POST	Stage at	ELISA	Southern	PCR	% injury	% injury	% injury
				PAINTED	N-1	N-2	over the top with 2,4-D (g ae/ha)	appl (# nodes)	(ng/mL)	Copy number	coding region	2 DAT	7 DAT	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									
					0=No Flip									

[00335] 11.2.4– Molecular Analyses: Soybean

[00336] 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.

[00337] 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 CTGTAAACGCC) (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.

[00338] 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.

[00339] 11.2.5 – Biochemical Analyses: Soybean

[00340] 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.

[00341] 11.2.6 – T₁ Progeny evaluation. T₀ plants will be allowed to self fertilize to derive T₁ 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. Surviving 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.

[00342] *AAD-12 (v1)* transgenic Maverick soybean plants have been generated thorough *Agrobacterium*- mediated cot-node transformation system. The T₀ 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.

[00343] 11.3 Transformation Method 2: Aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00344] 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.

[00345] 11.4 Transformation Method 3. Biolistic bombardment of Soybean

[00346] 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 soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

[00347] 11.5 Transformation Method 4. Whiskers mediated transformation.

[00348] 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 soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

[00349] 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% phytigel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium.

[00350] 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). Regenerated plants can also be assessed according to Example 11.2.

Example 12– *AAD-12 (v1)* in Cotton

[00351] 12.1 – Cotton transformation protocol.

[00352] 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 $\mu\text{E}/\text{m}^2$, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00353] 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.

[00354] 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.

[00355] 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 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).

[00356] 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								
Ingredient	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (200 ml)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B	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	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

12.2 – Cell transformation.

[00357] 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.

[00358] 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

[00359] 12.3 – Plant regeneration.

[00360] *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 T₁ seed or outcrossed with an elite cotton line to produce F₁ 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 traits as described in experiments 18, 19, 22, and 23.

Example 13 – *Agrobacterium* Transformation of Other Crops

[00361] 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 transformation of rye, *see, e.g.*, Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.*, Hinchey *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.

[00362] 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 tuberosum*), Sweet potato (*Ipomoea batatas*), 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 sativa*), 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*, *Cynodon*, and other genera), Clover (*Trifolium*), Vetch (*Vicia*). Such plants, with AAD-12 (v1) genes, for example, are included in the subject invention.

[00363] 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

[00364] 14.1 – AAD-2 (v1) initial cloning.

[00365] 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.

[00366] 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 (SEQ ID NO:16): 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 (SEQ ID NO:17): 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)].

[00367] 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), H₂O 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.

[00368] 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.

[00369] 14.2 – Completion of *AAD-2 (v1)* binary vector.

[00370] 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*.

[00371] 14.3 – Evaluation of transformed *Arabidopsis*.

[00372] Freshly harvested T₁ 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).

[00373] *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 T₁ *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*.

[00374] 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 T₁ 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

[00375] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

[00376] 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 Agrilience 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 mixes of 70-1120 or 35-560 g ae/ha of triclopyr and fluroxypyr, respectively, to increase spectrum and/or increase the ability 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)*

[00377] *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.

[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, 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.

[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 chloracetamide 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] 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.

[00381] 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC 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

[00382] 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.

[00383] 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.

[00384] 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.

[00385] 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 HTC 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

[00386] 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. Triclopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimen. 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.

[00387] 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC's 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

[00388] 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 (Agrilience, 2005).

[00389] 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 canadensis*) 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 g 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.

[00390] 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC 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

[00391] 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 (Agrilience, 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)*, monocot 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 multiple 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.

[00392] 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC 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

[00393] 21.1 – Media description.

[00394] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/L Phytigel (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).

[00395] 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).

[00396] 21.2 – Tissue culture development.

[00397] 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.

[00398] 21.3 – Microprojectile bombardment.

[00399] 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).

[00400] 21.4 – Postemergence herbicide tolerance in *AAD-12 (v1)* transformed T₀ Rice

[00401] 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.

[00402] 21.5 – Heritability of AAD-12 (v1) in T₁ rice

[00403] A 100-plant progeny test was conducted on five T₁ 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.

[00404] 21.6 – Verification of high 2,4-D tolerance in T₁ Rice.

[00405] The following T₁ *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.

[00406] 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 <i>AAD-12 (v1)</i> and untransformed control response to varying levels of 2,4-D DMA.			
	Lemont Untransformed Control	pDAB4101(20)003	pDAB4101(27)002
Herbicide	Average % Injury 14 DAT		
1120 g ac/ha 2,4-D DMA	20	10	10
2240 g ac/ha 2,4-D DMA	35	15	30
4480 g ac/ha 2,4-D DMA	50	23	40

[00407] 21.7 – Tissue harvesting, DNA isolation and quantification.

[00408] 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 fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

[00409] 21.8 – AAD-12 (v1) expression.

[00410] Sample preparation and analysis conditions were as described previously. All 33 T₀ 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.

[00411] 21.9– Field tolerance of pDAB4101 rice plants to 2,4-D and triclopyr herbicides.

[00412] 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 T₁ 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.

[00413] *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 excellent tolerance to the 2X rates of triclopyr with no injury observed at either 7 or 15DAT.

[00414] 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			
Active Ingredient	Rate	7DAT		15DAT	
		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

[00415] 22.1 – Canola transformation.

[00416] 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).

[00417] 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.

[00418] 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.

[00419] 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.

[00420] 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.

[00421] 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 Convicon for 1-2 weeks before transfer to the greenhouse.

[00422] 22.2 – Molecular analysis: canola materials and methods

[00423] 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.

[00424] 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.

[00425] 22.2.3 – ELISA.

[00426] 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 <i>AAD-12 (v1)</i> 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	-

[00427] 22.4 – Postemergence herbicide tolerance in *AAD-12(v1)* transformed T₀ Canola.

[00428] Forty-five T₀ 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.

[00429] 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 T₁ (and T₂ generation) by selfpollination under controlled, bagged, conditions.

[00430] 22.5 – AAD-12 (v1) heritability in Canola.

[00431] A 100 plant progeny test was also conducted on 11 T₁ 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.

[00432] 22.6 – Verification of high 2,4-D tolerance in T₁ Canola

[00433] For T₁ 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.

[00434] 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 T₁ 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 <i>AAD-12</i> (<i>v1</i>) and untransformed control response to varying rates postemergence 2,4-D DMA applications.						
	Untransformed Control	pDAB3759(33)013.001	pDAB3759(18)009.001	pDAB3759(18)022.001	pDAB3759(18)030.001	pDAB3759(18)023.001
Herbicide	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

[00435] 22.7-Field tolerance of pDAB3759 canola plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

[00436] 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)030.001, 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.

[00437] 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.

[00438] 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.

<u>Herbicide Treatment</u>		<u>% Visual Injury at 14 DAT</u>		
Active Ingredient	Rate	<i>AAD-12</i> event 3759(20)018.001	<i>AAD-12</i> 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

[00439] Insect resistance in crops supplied by a transgenic trait is prevalent 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.

[00440] 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 Agrilience (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

[00441] 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.

[00442] 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 the 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 as 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*.

[00443] 24.1—Cell culture--Alternative auxins.

[00444] *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.

[00445] 24.1.1—Tobacco cell suspension—2,4-D as selection agent.

[00446] 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, 100, 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.

[00447] 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.

[00448] 24.1.2—Tobacco cell transformation with *AAD-12 (v1)* and 2,4-D selection.

[00449] 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 OD⁶⁶⁰ were mixed with 100 ul of *Agrobacterium* (either EHA101 or LBA4404 strains) suspension at 1.0 OD⁶⁶⁰ 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.

[00450] 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.

[00451] 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, and 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.

[00452] 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.

[00453] 24.2.1— Cotton explants—auxin alternatives

[00454] 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-dichloroprop, 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.

[00455] R-dichloroprop 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.

[00456] 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 cotyledonary 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. An expression cassette comprising a polynucleotide operably linked to a promoter that is functional in a plant cell, wherein said polynucleotide encodes a protein that catalyzes degradation of a herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.
2. The expression cassette of claim 1, wherein said polynucleotide comprises plant codons for expression in said plant cell.
3. The expression cassette of claim 1, wherein said promoter is 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.
4. A method of controlling herbicide resistance in weeds in a field, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of crop plants comprising an expression cassette of claim 1.
5. A method of selecting at least one transgenic plant that is resistant or tolerant to both phenoxy auxin and pyridyloxy auxin herbicides, said method comprising applying a herbicidically effective amount of aryloxyalkanoate herbicide to a plurality of plants in a field, wherein said plurality of plants in said field comprising at least one plant that is stably transformed with the expression cassette of claim 1 and is able to grow in said effective amount of aryloxyalkanoate herbicide, and identifying said plant that is stably transformed with said expression cassette .

6. The method of claim 4, wherein said plurality of crop plants are dicots.
7. The method of claim 6, wherein said dicots are selected from the group consisting of soybean plants, cotton plants, canola plants, sugar beets plants, tomato, and sunflower.
8. The method of claim 4, wherein said pyridyloxy auxin herbicide is one or more pyridyloxyacetate herbicides.
9. The method of claim 8, wherein said pyridyloxyacetate herbicide is selected from the group of triclopyr and fluroxypyr.
10. The method of claim 4, wherein said method comprises applying said herbicide to said crop field within 14 days planting a seed in said crop field, wherein said seed comprises the expression cassette of claim 1.
11. The method of claim 4, wherein said plurality of crop plants further comprise a second heterologous polynucleotide encoding a second protein that confers said crop plants with resistance to at least one other herbicide, and said method further comprising applying said at least one other herbicide to least a portion of said field.
12. The method of claim 11, wherein said herbicides are applied sequentially or concurrently.
13. The method of claim 11, wherein said herbicides are applied from a tank mix.
14. The method of claim 11, 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, pyriithiobac, pyroxasulfone, pyroxsulam, quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxydim, terbacil, thiazopyr, thidiazuron, thiencazone, thifensulfuron, thiobencarb, topramezone, tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflusulfuron, and tritosulfuron.

15. The method of claim 4, wherein said weeds are glyphosate-resistant.
16. The method of claim 15, wherein said crop plants are glyphosate tolerant crop plants.
17. The method of claim 4, wherein said herbicide is a phenoxy auxin.
18. The method of claim 17, wherein said phenoxy auxin is selected from the group consisting of MCPA and a 2,4-D herbicide.
19. A method of controlling volunteer plants in a field, wherein said method comprises applying to said volunteer plants at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of transgenic crop plants comprising an expression cassette of claim 1.
20. The method of claim 19 wherein said volunteer plants are glyphosate-resistant volunteer and of a different species than said transgenic crop plants.

21. 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.
22. 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.
23. The polynucleotide of claim 22 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.
24. 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.
25. The polynucleotide of claim 24 wherein said promoter is a plant promoter.
26. The polynucleotide of claim 25 wherein said promoter is a cassava vein mosaic virus promoter.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)		Attorney Docket Number	DAS-P0129-04-US-E
		First Named Inventor	Terry R. Wright
		COMPLETE IF KNOWN	
☐ Declaration Submitted With Initial Filing	OR	☐ Declaration Submitted After Initial Filing (surcharge (37 CFR 1.16(f)) required)	Application Number
			Filing Date
			Art Unit
			Examiner Name

I hereby declare that: (1) Each inventor's residence, mailing address, and citizenship are as stated below next to their name; and (2) I believe the inventor(s) named below to be the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention titled:

NOVEL HERBICIDE RESISTANCE GENES

(Title of the Invention)

the application of which was made or was authorized to be made by me and

☒ is attached hereto

OR

☐ was filed on (MM/DD/YYYY) _____ as United States Application Number or PCT International
Application Number _____ and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment specifically 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.

Authorization To Permit Access To Application by Participating Offices

☐ If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the above-identified patent application is filed access to the above-identified patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the above-identified patent application is filed to have access to the above-identified patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the above-identified patent application with respect to: 1) the above-identified patent application-as-filed; 2) any foreign application to which the above-identified patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the above-identified patent application; and 3) any U.S. application-as-filed from which benefit is sought in the above-identified patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing the Authorization to Permit Access to Application by Participating Offices.

[Page 1 of 3]

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. 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 21 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.**

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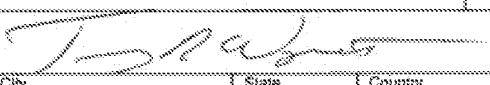
DECLARATION — Utility or Design Patent Application

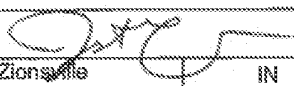
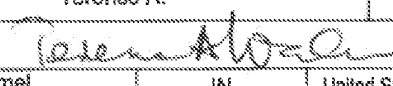
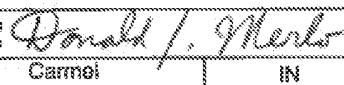
Claim of Foreign Priority Benefits

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(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 rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

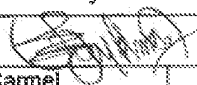
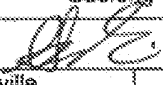
Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application number(s) are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

DECLARATION — Utility or Design Patent Application			
Direct all correspondence to:		☒ The address associated with Customer Number: 88067	OR ☐ Correspondence address below
Name			
Address			
City		State	ZIP
Country	Telephone	Email	
<p style="text-align: center;">WARNING:</p> <p>Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.</p> <p>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.</p> <p>I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) year, or both.</p>			
NAME OF SOLE OR FIRST INVENTOR:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Terry R.		Wright	
Inventor's Signature		Date	
		7-31-2014	
Residence: City	State	Country	Citizenship
Carmel	IN	United States of America	US
Mailing Address: 14162 Charity Chase Circle			
City	State	Zip	Country
Carmel	IN	46074	United States of America
☒ Additional inventors or a legal representative are being named on the <u>2</u> supplemental sheet(s) PTO/SB/02A or 02L/R attached hereto.			

DECLARATION		ADDITIONAL INVENTOR(S) Supplemental Sheet	
		Page 1 of 2	
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Justin M.		Lira	
Inventor's Signature 		Date <u>Aug 6, 2014</u>	
Residence: City	State	Country	Citizenship
Zionsville	IN	United States of America	US
Mailing Address: 8974 Windpointe Pass			
City	State	Zip	Country
Zionsville	IN	46077	United States of America
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Terence A.		Walsh	
Inventor's Signature 		Date <u>August 6, 2014</u>	
Residence: City	State	Country	Citizenship
Carmel	IN	United States of America	US
Mailing Address: 12443 Branford Street			
City	State	Zip	Country
Carmel	IN	46032	United States of America
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Donald J.		Merlo	
Inventor's Signature 		Date <u>July 31, 2014</u>	
Residence: City	State	Country	Citizenship
Carmel	IN	United States of America	US
Mailing Address: 11845 Durbin Drive			
City	State	Zip	Country
Carmel	IN	46032	United States of America

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION				ADDITIONAL INVENTOR(S) Supplemental Sheet	
				Page 2 of 2	
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Jayakumar Pon			Samuel		
Inventor's Signature 			Date 08/18/2014		
Residence: City Carmel	State IN	Country United States of America	Citizenship US		
Mailing Address 3336 Kilkenny Circle					
City Carmel	State IN	Zip 46032	Country United States of America		
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Gaofeng			Lin		
Inventor's Signature 			Date August 10, 2014		
Residence: City Zionsville	State IN	Country United States of America	Citizenship US		
Mailing Address 981 Franklin Trace					
City Zionsville	State IN	Zip 46077	Country United States of America		
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Inventor's Signature			Date		
Residence: City	State	Country	Citizenship		
Mailing Address:					
City	State	Zip	Country		

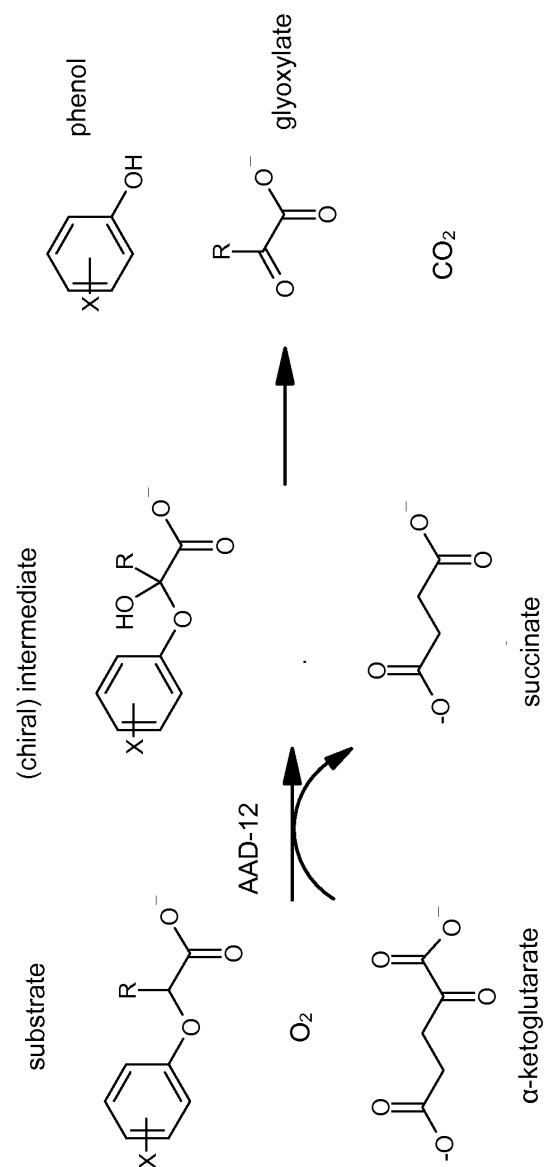


FIG. 1

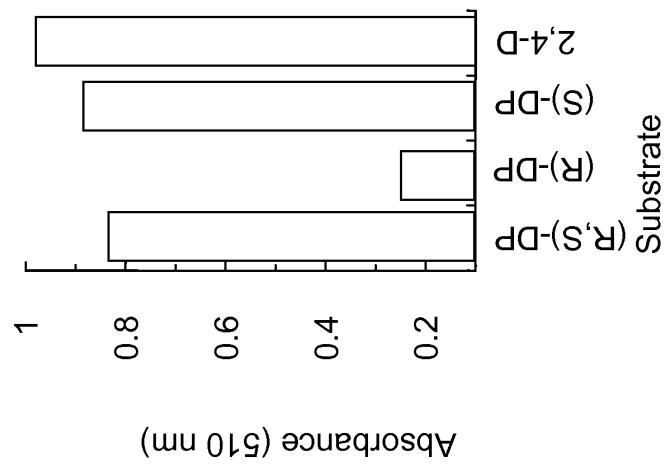


FIG. 3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Ref. : DAS-P0129-04-US-E
Applicants : Terry R. Wright *et al.*
Serial No. : N/A
Conf. No. : N/A
Filed : Concurrent filing
Examiner : N/A
Title : NOVEL HERBICIDE RESISTANCE GENES

STATEMENT IN SUPPORT OF SEQUENCE LISTING

Pursuant to 37 C.F.R. § 1.821 (c), (e), and (f), Applicant states that the herein submitted sequence listing in computer readable form (.txt file) provides the same information as the sequence listing filed with the parent case USSN 13/647,081.

If the Authorizing Officer has any questions regarding this submission, the Officer is invited to contact Mr. Jay Sanders, at 317-237-1245, or the undersigned.

/yonghao hou/

Yonghao Hou, Reg. # 61,391
Faegre Baker Daniels LLP
300 N. Meridian Street, Suite 2700
Indianapolis, Indiana 46204
Telephone: (317) 237-1469
Fax: (317) 237-1000

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:		Yonghao Hou		
Attorney Docket Number:		DAS-P0129-04-US-E		
Filed as Large Entity				
Utility under 35 USC 111(a) Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Utility application filing	1011	1	280	280
Utility Search Fee	1111	1	600	600
Utility Examination Fee	1311	1	720	720
Pages:				
Claims:				
Claims in Excess of 20	1202	6	80	480
Independent claims in excess of 3	1201	1	420	420
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				2500

Electronic Acknowledgement Receipt	
EFS ID:	20191032
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	83067
Filer:	Yonghao Hou
Filer Authorized By:	
Attorney Docket Number:	DAS-P0129-04-US-E
Receipt Date:	19-SEP-2014
Filing Date:	
Time Stamp:	14:59:05
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$2500
RAM confirmation Number	1289
Deposit Account	020390
Authorized User	
<p>The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:</p> <p>Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)</p>	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	DAS-P0129-04-ADS.pdf	1561968	no	8
			a395fab5ad0f40a9e876d117cc0a6a1c24a4824		
Warnings:					
Information:					
2		DAS-P0129-04-APP.pdf	951159	yes	145
			540c2378131630e69389e725bea94bd5e09bf9e		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	141	
	Claims		142	144	
	Abstract		145	145	
Warnings:					
Information:					
3	Oath or Declaration filed	DAS-P0129-04-DEC.pdf	329765	no	5
			35a808b7fb38d77603c23f73abbd0bc025efba2c		
Warnings:					
Information:					
4	Drawings-only black and white line drawings	DAS-P0129-04-DRAW.pdf	64012	no	3
			5bd0ded58354a707d9d0c4f775d6bc69c6617890		
Warnings:					
Information:					
5	Sequence Listing	DAS-P0129-04-SEQSTATEMENT.pdf	87943	no	1
			098f7b71e0aa4211e993d172fd393218b31d6bfa		
Warnings:					
Information:					
6	Sequence Listing (Text File)	DAS-P0129-04-SEQLISTING.txt	22009	no	0
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	37938	no	2
			434117b0fdc319b4f41638ad7d71d617e3114150		

Warnings:	
Information:	
Total Files Size (in bytes):	3054794
<p>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.</p> <p><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.</p> <p><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.</p> <p><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.</p>	

=====
Sequence Listing could not be accepted.

If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).

Reviewer: Kathleen Kalafus

Timestamp: [year=2014; month=9; day=19; hr=16; min=21; sec=4; ms=342;]
=====

Reviewer Comments:

1.

W402 Undefined organism found in <213> in SEQ ID (21)

Numeric identifier <213> can only be one of three choices: Scientific
name (i.e. Genus/species), Unknown, or Artificial Sequence.

The entry in the <213> field of SEQ ID NO: 21, "bacteria" is
insufficient. Please enter a proper genus/species name. If the
genus/species name is not known, please use "unknown" in <213>.

When using "Unknown", for numeric identifier <213>, a mandatory feature
is required to explain the source of the genetic material. The feature
consists of numeric identifier <220>, which remains blank and numeric
identifier <223>, which states the source of the genetic material. The
use of "Unknown", for numeric identifier <213>, indicates that the
sequence is not artificial and the "Genus/Species" is not known.
Because the "Genus/Species" is not known it is therefore acceptable to
provide as much taxonomic information as possible about the organism
from which the genetic material was extracted. For instance, if all
that is known about the source of the genetic material is that it is
"bacterial" or "mammalian," it is acceptable to use these terms as the
source in the mandatory feature. When the origin of the genetic
information is completely unknown, it is acceptable to explain where the
sample was taken, such as a soil sample. Please make all necessary
changes.

2.

W402	Undefined organism found in <213> in SEQ ID (1)
W402	Undefined organism found in <213> in SEQ ID (2)
W402	Undefined organism found in <213> in SEQ ID (3)
W402	Undefined organism found in <213> in SEQ ID (4)
W402	Undefined organism found in <213> in SEQ ID (5)
W213	Artificial or Unknown found in <213> in SEQ ID (6)
W213	Artificial or Unknown found in <213> in SEQ ID (7)
W213	Artificial or Unknown found in <213> in SEQ ID (8)
W213	Artificial or Unknown found in <213> in SEQ ID (9)
W213	Artificial or Unknown found in <213> in SEQ ID (10)
W213	Artificial or Unknown found in <213> in SEQ ID (11)
W213	Artificial or Unknown found in <213> in SEQ ID (12)
W213	Artificial or Unknown found in <213> in SEQ ID (13)
W213	Artificial or Unknown found in <213> in SEQ ID (14)
W213	Artificial or Unknown found in <213> in SEQ ID (15)
W213	Artificial or Unknown found in <213> in SEQ ID (16)
W213	Artificial or Unknown found in <213> in SEQ ID (17)
W402	Undefined organism found in <213> in SEQ ID (18)
W402	Undefined organism found in <213> in SEQ ID (19)
W402	Undefined organism found in <213> in SEQ ID (20)

Note:

The errors listed above are exemplary and may not be a complete list of all errors in the sequence listing file. The Applicant is required to review the sequence listing in its entirety and correct all instances of similar errors.

To correct the sequence listing errors noted in this report - The recommended method for correction of errors is to access the sequence listing working file using the software program in which the listing was originally prepared, e.g., the project file in PatentIn, make any necessary corrections within that program, then generate a new sequence listing file. Use of a word processing program to correct errors directly in the original sequence listing file is strongly discouraged, since such programs often introduce unintended changes to the sequence listing, rendering the listing unacceptable. When the working file or original program is not available for correction, then use of a common or plain text-only editor, such as NotePad, to edit the original sequence listing file may suffice.

A sequence listing text file submitted via EFS-Web that complies with the requirements of 37 CFR 1.824(a) (2)-(6) and (b) (i.e., is a compliant sequence listing ASCII text file), serves as both the paper copy required by 37 CFR 1.821(c) and the CRF required by 37 CFR 1.821(e).

Application No: 14491197 Version No: 1.0

Input Set:

Output Set:

Started: 2014-09-19 15:00:49.810
 Finished: 2014-09-19 15:00:50.964
 Elapsed: 0 hr(s) 0 min(s) 1 sec(s) 154 ms
 Total Warnings: 21
 Total Errors: 0
 No. of SeqIDs Defined: 21
 Actual SeqID Count: 21

Error code	Error Description
W 402	Undefined organism found in <213> in SEQ ID (1)
W 402	Undefined organism found in <213> in SEQ ID (2)
W 402	Undefined organism found in <213> in SEQ ID (3)
W 402	Undefined organism found in <213> in SEQ ID (4)
W 402	Undefined organism found in <213> in SEQ ID (5)
W 213	Artificial or Unknown found in <213> in SEQ ID (6)
W 213	Artificial or Unknown found in <213> in SEQ ID (7)
W 213	Artificial or Unknown found in <213> in SEQ ID (8)
W 213	Artificial or Unknown found in <213> in SEQ ID (9)
W 213	Artificial or Unknown found in <213> in SEQ ID (10)
W 213	Artificial or Unknown found in <213> in SEQ ID (11)
W 213	Artificial or Unknown found in <213> in SEQ ID (12)
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W 213	Artificial or Unknown found in <213> in SEQ ID (17)
W 402	Undefined organism found in <213> in SEQ ID (18)
W 402	Undefined organism found in <213> in SEQ ID (19)
W 402	Undefined organism found in <213> in SEQ ID (20)

Input Set:

Output Set:

Started: 2014-09-19 15:00:49.810
Finished: 2014-09-19 15:00:50.964
Elapsed: 0 hr(s) 0 min(s) 1 sec(s) 154 ms
Total Warnings: 21
Total Errors: 0
No. of SeqIDs Defined: 21
Actual SeqID Count: 21

Error code	Error Description
W 402	Undefined organism found in <213> in SEQ ID (21)

SEQUENCE LISTING

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<120> Novel Herbicide Resistance Genes

<130> DAS-P0129-02-US-e

<140> US 14/491,197

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gcggtgttct cggccgaagt ggtgcccgca gtgggcgggc gcacctgctt cggcgacatg	420
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 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
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Gly Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe
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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
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Val Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala
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Tyr Asp Ala Leu Asp Glu Ala Thr Arg Ala Leu Val His Gln Arg Ser
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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
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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
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245 250 255

Asp Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe Lys Leu
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caacatgctc tcttgatttt cccaggtcag cacctgtcca acgaccagca aatcactttt 180
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35 40 45

Pro Leu Ser Gln Asp Gln Gln Ile Ala Phe Ala Arg Asn Phe Gly Pro
50 55 60

Leu Glu Gly Gly Phe Ile Lys Val Asn Gln Arg Pro Ser Arg Phe Lys
65 70 75 80

Tyr Ala Glu Leu Ala Asp Ile Ser Asn Val Ser Leu Asp Gly Lys Val
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Ala Gln Arg Asp Ala Arg Glu Val Val Gly Asn Phe Ala Asn Gln Leu
100 105 110

Trp His Ser Asp Ser Ser Phe Gln Gln Pro Ala Ala Arg Tyr Ser Met
115 120 125

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130 135 140

Asp Met Arg Ala Ala Tyr Asp Ala Leu Pro Arg Asp Leu Gln Ser Glu
145 150 155 160

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165 170 175

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Val Asn Trp Pro Leu Val Arg Thr His Ala Gly Ser Gly Arg Lys Phe
195 200 205

Leu Phe Ile Gly Ala His Ala Ser His Val Glu Gly Leu Pro Val Ala
210 215 220

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225 230 235 240

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65 70 75 80

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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
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 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
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 Val

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 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

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 09/24/2014

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO.	TOT CLAIMS	IND CLAIMS
14/491,197	09/19/2014	1638	2900	DAS-P0129-04-US-E	26	4

CONFIRMATION NO. 3336

83067

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204

FILING RECEIPT



OC000000070972691

Date Mailed: 10/02/2014

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Terry R. Wright, Carmel, IN;
Justin M. Lira, Zionsville, IN;
Terence Anthony Walsh, Zionsville, IN;
Donald Merlo, Carmel, IN;
Pon Samuel Jayakumar, Carmel, IN;
Gaofeng Lin, Zionsville, IN;

Applicant(s)

Dow AgroSciences LLC, Indianapolis, IN

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 13/647,081 10/08/2012

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 09/24/2014

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/491,197**

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No

page 1 of 3

Title

NOVEL HERBICIDE RESISTANCE GENES

Preliminary Class

800

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 14/491,197	
APPLICATION AS FILED - PART I							
(Column 1)		(Column 2)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE(\$)	FEE(\$)			
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A				
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A				
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A				
TOTAL CLAIMS (37 CFR 1.16(i))	26	minus 20 = 6			OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	4	minus 3 = 1			OR		
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 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).				OR		
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					OR		
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL		OR	
						OR	
APPLICATION AS AMENDED - PART II							
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY	
AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)	OR OTHER THAN SMALL ENTITY	
	Total (37 CFR 1.16(i))	*	Minus **	=	x	=	OR
	Independent (37 CFR 1.16(h))	*	Minus ***	=	x	=	OR
	Application Size Fee (37 CFR 1.16(s))					OR	
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					OR	
				TOTAL ADD'L FEE		OR	
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY	
AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)	OR OTHER THAN SMALL ENTITY	
	Total (37 CFR 1.16(i))	*	Minus **	=	x	=	OR
	Independent (37 CFR 1.16(h))	*	Minus ***	=	x	=	OR
	Application Size Fee (37 CFR 1.16(s))					OR	
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					OR	
				TOTAL ADD'L FEE		OR	
<p>* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.</p> <p>** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".</p> <p>*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".</p> <p>The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.</p>							



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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

IMPROPER CFR REQUEST



OC000000070988065

Date Mailed: 10/02/2014

83067

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204

RESPONSE TO REQUEST FOR CORRECTED FILING RECEIPT

Continuity, Priority Claims, Petitions, and Non-Publication Requests

In response to your request for a corrected Filing Receipt, the Office is unable to comply with your request because:

Improper Benefit Claim(s) to Prior-Filed Nonprovisional Application(s)

The benefit claim(s) to prior-filed nonprovisional application(s) is improper because there is no specific reference for each prior-filed application that includes: (1) the identification of the prior-filed application by application number, or international application number and international filing date; and (2) a clear indication of the relationship (i.e., continuation, divisional, or continuation-in-part) of the nonprovisional applications. Applicant must submit a corrected application data sheet (ADS) to include a specific reference. Applicant should review each benefit claim submitted and, if appropriate, provide the proper reference to the prior-filed application(s) as required by 37 CFR 1.78. A proper relationship includes an identification of each nonprovisional application as a continuation, divisional, or continuation-in-part application of the immediate prior-filed nonprovisional application for which a benefit is claimed under 37 CFR 1.78 in order to establish copendency throughout the entire chain of prior-filed applications. The specific reference must be included in the domestic benefit information section of an application data sheet (37 CFR 1.76).

Timeliness: The required reference for each benefit claim must be filed during the pendency of the instant application and within the later of: (1) four months from the actual filing date of the instant application, or the national stage commencement date if the instant application is a national stage application under 35 U.S.C. 371; or (2) sixteen months from the filing date of the prior-filed application. Failure to timely file the required reference is considered a waiver of any benefit claim, unless a grantable petition to accept an unintentionally delayed claim under 37 CFR 1.78(a), the surcharge set forth in 37 CFR 1.17(t), and the required reference are filed. See 37 CFR 1.78(a).

/kgebremichael/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101



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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

FORMALITIES LETTER

83067

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204



OC00000070972692

Date Mailed: 10/02/2014

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- An abstract of the technical disclosure preferably not exceeding 150 words in length and commencing on a separate sheet in compliance with 37 CFR 1.72(b) is required. An abstract was not provided for this application.
- A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 CFR 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing." Applicant must provide a substitute computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). Refer to attachment or PAIR document dated 9/19/14.

For questions regarding compliance to these requirements, please contact:

- For Rules interpretation, call (571) 270-5300
- For CRF submission help, call (571) 272-2510
- For PatentIn software program support technical assistance, call (866)217-9197.
- Send e-mail correspondence for PatentIn software program help to ebc@uspto.gov
- Patent software is available at <http://www.uspto.gov/patents/resources/tools/checker/patentinrel.jsp> .

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.

The surcharge is due for any one of:

- late submission of the basic filing fee, search fee, or examination fee,
- late submission of inventor's oath or declaration,
- filing an application that does not contain at least one claim on filing, or
- submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- \$ **140** surcharge.
- \$(**0**) previous unapplied payment amount.
- \$ **140** TOTAL FEE BALANCE DUE.

Items Required To Avoid Processing Delays:

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
Pon Samuel Jayakumar

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice".
<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.

/kgebremichael/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

To: inteas@faegrebd.com,cynthia.payson@faegrebd.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 83067

Oct 02, 2014 05:35:44 AM

Dear PAIR Customer:

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 83067 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:

The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
14491197	APP.FILE.REC	10/02/2014	DAS-P0129-04-US-E
	M327	10/02/2014	DAS-P0129-04-US-E
	NT.CR.APP.PA	10/02/2014	DAS-P0129-04-US-E

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>Attorney</i>	}	<i>Confirmation No.</i> 3336
<i>Docket No.:</i> DAS-P0129-04-US-E	}	
	}	
<i>Applicant:</i> Terry R. Wright et al.	}	
	}	
<i>Invention:</i> NOVEL HERBICIDE RESISTANCE GENES	}	
	}	
<i>Serial No:</i> 14/491,197	}	
	}	
<i>Filed:</i> September 19, 2014	}	
	}	
	}	

REQUEST FOR CORRECTION OF FILING RECEIPT and
SUPPLEMENTAL APPLICATION DATA SHEET, CORRECTED APPLICATION
PAPERS

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

Sir:

A revised application data sheet is attached to this Request to list proper benefit claim(s) to prior-filed non-provisional application(s) in page 4, and to correct the name of the 5th inventor.

Thus, it is requested that the filing receipt be corrected as indicated on the enclosed copy thereof. Specifically, the name of the 5th inventor should be listed as:

Jayakumar Pon Samuel

In light of his name correction in the revised application data sheet, the oath/declaration of inventor Jayakumar Pon Samuel is proper, because his correct name and signature are properly included in page 5 of the oath/declaration submitted on September 19, 2014. The requirement of submitting his executed oath/declaration is moot.

In the notification received on Oct. 2, 2014, the applicant is requested filing corrected application papers including sequence listing and Abstract. The sequence listing in its computer readable format with statement in support thereof are submitted concurrently. An abstract is included.

It is requested that a corrected filing receipt be forwarded to the undersigned. If there are any fees due with the filing of this document, the undersigned authorizes the appropriate fee be withdrawn from deposit account number 02-0390.

Respectfully submitted,

/yonghao hou /

Yonghao Hou, Reg. No. 61,391

Attorney for Applicant

Faegre Baker Daniels LLP

Customer No. 83067

Attachments:

Corrected ADS

Filing Receipt Redline

Corrected Sequence listing and statement of support

Oath/Declaration Filed

Abstract

Notice to File Corrected Application Papers

2014-12-02



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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

FORMALITIES LETTER

83067
Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204



Date Mailed: 10/02/2014

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- An abstract of the technical disclosure preferably not exceeding 150 words in length and commencing on a separate sheet in compliance with 37 CFR 1.72(b) is required. An abstract was not provided for this application.
- A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 CFR 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing." Applicant must provide a substitute computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). Refer to attachment or PAIR document dated 9/19/14.

For questions regarding compliance to these requirements, please contact:

- For Rules interpretation, call (571) 270-5300
- For CRF submission help, call (571) 272-2510
- For PatentIn software program support technical assistance, call (866)217-9197.
- Send e-mail correspondence for PatentIn software program help to ebc@uspto.gov
- Patent software is available at <http://www.uspto.gov/patents/resources/tools/checker/patentinrel.jsp> .

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.

The surcharge is due for any one of:

- late submission of the basic filing fee, search fee, or examination fee,
- late submission of inventor's oath or declaration,
- filing an application that does not contain at least one claim on filing, or
- submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- \$ **140** surcharge.
- \$(**0**) previous unapplied payment amount.
- \$ **140** TOTAL FEE BALANCE DUE.

Items Required To Avoid Processing Delays:

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
Pon Samuel Jayakumar

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice".
<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.

/kgebremichael/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

Electronic Patent Application Fee Transmittal				
Application Number:		14491197		
Filing Date:		19-Sep-2014		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry R. Wright		
Filer:		Yonghao Hou		
Attorney Docket Number:		DAS-P0129-04-US-E		
Filed as Large Entity				
Utility under 35 USC 111(a) Filing Fees				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Late Filing Fee for Oath or Declaration	1051	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
Total in USD (\$)				140

Electronic Acknowledgement Receipt	
EFS ID:	20840575
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	83067
Filer:	Yonghao Hou
Filer Authorized By:	
Attorney Docket Number:	DAS-P0129-04-US-E
Receipt Date:	02-DEC-2014
Filing Date:	19-SEP-2014
Time Stamp:	14:55:26
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$ 140
RAM confirmation Number	1167
Deposit Account	020390
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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1	Miscellaneous Incoming Letter	DAS-P0129-04-SEQSTATEMENT12-2-14.pdf	92855 b9f96e24c8107c77c23d1ae78f5eb6e1ddfd e829	no	1
Warnings:					
Information:					
2	Request for Corrected Filing Receipt	DAS-P0129-04-FRRED.pdf	176416 271b18808877cdcd5483cd6de1293712 4c3832	no	3
Warnings:					
Information:					
3	Abstract	DAS-P0129-04-ABSTRACT.pdf	27723 dbf8c7e89d1a26319008614dc5662da57ab b2440	no	1
Warnings:					
Information:					
4	Oath or Declaration filed	DAS-P0129-04-DEC12-1-14.pdf	310649 3d543df28d61c3f80b76f9892465097bb5 8cc68	no	4
Warnings:					
Information:					
5	Sequence Listing (Text File)	DAS-P0129-04-SEQCorrectedST25.txt	22030	no	0
Warnings:					
Information:					
6	Application Data Sheet	DAS-P0129-04-ADSREV12-2-14.pdf	124667 aae36f6f8cc75ad184b6c767f2538b1811d5 d188	no	8
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
7	Applicant Response to Pre-Exam Formalities Notice	DAS-P0129-04-RESPMP.pdf	199353 86e0191262a08eff69b1fb8e36eb84904958 4a17	no	4
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	29918 d84de1273efaa1458dbcd43e20baeabbcd 41027	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			983611		

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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Ref. : DAS-P0129-04-US-E
Applicants : Terry R. Wright *et al.*
Serial No. : 14/491,197
Conf. No. : 3336
Filed : 9/19/2014
Examiner : N/A
Title : NOVEL HERBICIDE RESISTANCE GENES

STATEMENT IN SUPPORT OF SEQUENCE LISTING

Pursuant to 37 C.F.R. § 1.822 and 1.823, Applicant states that the herein submitted sequence listing in computer readable form (.txt file) provides the same information as the sequence listing filed for the above identified application, with corrected sequence source for SEQ ID NO:21, as directed by Ms. Kathleen KALAFUS from USPTO scientific and technical information centre. This submission complies 37 CFR § 1.821 (e), (f) (g) or 1.825 (b) (d), and includes no new matter.

If the Authorizing Officer has any questions regarding this submission, the Officer is invited to contact Mr. Jay Sanders, at 317-237-1245, or the undersigned.

/yonghao hou/

Yonghao Hou, Reg. # 61,391
Faegre Baker Daniels LLP
300 N. Meridian Street, Suite 2700
Indianapolis, Indiana 46204
Telephone: (317) 237-1469
Fax: (317) 237-1000



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

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO.	TOT CLAIMS	IND CLAIMS
14/491,197	09/19/2014	1638	2900	DAS-P0129-04-US-E	26	4

CONFIRMATION NO. 3336

83067

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204

FILING RECEIPT



0000000070972691

Date Mailed: 10/02/2014

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Terry R. Wright, Carmel, IN;
Justin M. Lira, Zionsville, IN;
Terence Anthony Walsh, Zionsville, IN;
Donald Merlo, Carmel, IN;
Pon Samuel Jayakumar, Carmel, IN; Jayakumar P. Samuel
Gaofeng Lin, Zionsville, IN;

Applicant(s)

Dow AgroSciences LLC, Indianapolis, IN

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 13/647,081 10/08/2012

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 09/24/2014

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/491,197**

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No

page 1 of 3

Title

NOVEL HERBICIDE RESISTANCE GENES

Preliminary Class

800

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.

Abstract

[00455] 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.

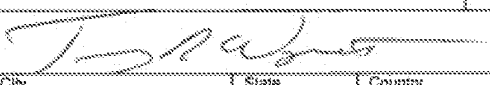
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION — Utility or Design Patent Application**Claim of Foreign Priority Benefits**

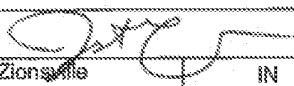
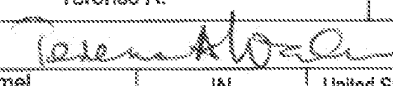
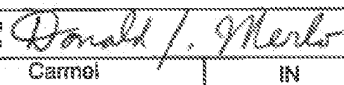
I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

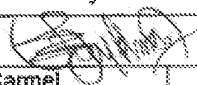
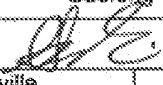
☐ Additional foreign application number(s) are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

DECLARATION — Utility or Design Patent Application			
Direct all correspondence to:		☒ The address associated with Customer Number: 88067	OR ☐ Correspondence address below
Name			
Address			
City		State	ZIP
Country	Telephone	Email	
<p style="text-align: center;">WARNING:</p> <p>Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.</p> <p>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.</p> <p>I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) year, or both.</p>			
NAME OF SOLE OR FIRST INVENTOR:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Terry R.		Wright	
Inventor's Signature		Date	
		7-31-2014	
Residence: City	State	Country	Citizenship
Carmel	IN	United States of America	US
Mailing Address: 14162 Charity Chase Circle			
City	State	Zip	Country
Carmel	IN	46074	United States of America
☒ Additional inventors or a legal representative are being named on the <u>2</u> supplemental sheet(s) PTO/SB/02A or 02L/R attached hereto.			

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION		ADDITIONAL INVENTOR(S) Supplemental Sheet	
		Page 1 of 2	
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Justin M.		Lira	
Inventor's Signature 		Date <u>Aug 6, 2014</u>	
Residence: City	State	Country	Citizenship
Zionsville	IN	United States of America	US
Mailing Address: 8974 Windpointe Pass			
City	State	Zip	Country
Zionsville	IN	46077	United States of America
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Terence A.		Walsh	
Inventor's Signature 		Date <u>August 6, 2014</u>	
Residence: City	State	Country	Citizenship
Carmel	IN	United States of America	US
Mailing Address: 12443 Branford Street			
City	State	Zip	Country
Carmel	IN	46032	United States of America
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Donald J.		Merlo	
Inventor's Signature 		Date <u>July 31, 2014</u>	
Residence: City	State	Country	Citizenship
Carmel	IN	United States of America	US
Mailing Address: 11845 Durbin Drive			
City	State	Zip	Country
Carmel	IN	46032	United States of America

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION				ADDITIONAL INVENTOR(S) Supplemental Sheet	
				Page 2 of 2	
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Jayakumar Pon			Samuel		
Inventor's Signature 			Date 08/18/2014		
Residence: City Carmel	State IN	Country United States of America	Citizenship US		
Mailing Address: 3336 Kilkeny Circle					
City Carmel	State IN	Zip 46032	Country United States of America		
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Gaofeng			Lin		
Inventor's Signature 			Date August 10, 2014		
Residence: City Zionsville	State IN	Country United States of America	Citizenship US		
Mailing Address: 981 Franklin Trace					
City Zionsville	State IN	Zip 46077	Country United States of America		
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Inventor's Signature			Date		
Residence: City	State	Country	Citizenship		
Mailing Address:					
City	State	Zip	Country		

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		
<p>The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.</p> <p>This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.</p>			

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Terry	R.	Wright		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Carmel	State/Province	IN	Country of Residenceⁱ	US
Mailing Address of Inventor:					
Address 1		14162 Charity Chase Circle			
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46074	Countryⁱ	US		
Inventor 2					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Justin	M.	Lira		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Zionsville	State/Province	IN	Country of Residenceⁱ	US
Mailing Address of Inventor:					
Address 1		8974 Windpointe Pass			
Address 2					
City	Zionsville	State/Province	IN		
Postal Code	46077	Countryⁱ	US		
Inventor 3					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Terence	Anthony	Walsh		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		DAS-P0129-04-US-E	
		Application Number			
Title of Invention		NOVEL HERBICIDE RESISTANCE GENES			
City	Zionsville	State/Province	IN	Country of Residence ⁱ	US
Mailing Address of Inventor:					
Address 1		11638 Promontory Trail			
Address 2					
City	Zionsville	State/Province	IN		
Postal Code	46077	Country ⁱ	US		
Inventor 4					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Donald		Merlo		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Carmel	State/Province	IN	Country of Residence ⁱ	US
Mailing Address of Inventor:					
Address 1		11845 Durbin Drive			
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46032	Country ⁱ	US		
Inventor 5					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Pon-Samuel- Jayakumar	P.	Jayakumar- Samuel		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Carmel	State/Province	IN	Country of Residence ⁱ	US
Mailing Address of Inventor:					
Address 1		3336 Kilkenny Circle			
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46032	Country ⁱ	US		
Inventor 6					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Gaofeng		Lin		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Zionsville	State/Province	IN	Country of Residence ⁱ	US

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E	
		Application Number		
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES			
Mailing Address of Inventor:				
Address 1	981 Franklin Trace			
Address 2				
City	Zionsville	State/Province	IN	
Postal Code	46077	Country ⁱ	US	
All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.				<input type="button" value="Add"/>

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).			
☐ An Address is being provided for the correspondence information of this application.			
Customer Number	83067		
Email Address	intear@faegrebd.com	<input type="button" value="Add Email"/>	<input type="button" value="Remove Email"/>

Application Information:

Title of the Invention	NOVEL HERBICIDE RESISTANCE GENES		
Attorney Docket Number	DAS-P0129-04-US-E	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	3	Suggested Figure for Publication (if any)	

Filing By Reference :

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)
Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing. ☐

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	☒ Customer Number	☐ US Patent Practitioner	☐ Limited Recognition (37 CFR 11.9)
Customer Number	83067		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

When referring to the current application, please leave the application number blank.

Prior Application Status	Pending		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
	Continuation of	13647081	2012-10-08		
Prior Application Status	Pending		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
13647081	Continuation of	12091896	2008-11-03		
Prior Application Status	Patented		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12091896	a 371 of international	PCTUS2006042133	2006-10-27	8283522	2012-10-09
Prior Application Status	Expired		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
PCTUS2006042133	Claims benefit of provisional	60731044	2005-10-28		
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.					

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)ⁱ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	<div>Remove</div> Access Code ⁱ (if applicable)
Additional Foreign Priority Data may be generated within this form by selecting the Add button.			

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

<p>This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.</p> <p>☐ NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.</p>
--

Authorization to Permit Access:

☐ Authorization to Permit Access to the Instant Application by the Participating Offices
<p>If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.</p> <p>In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.</p> <p>In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.</p>

Applicant Information:

<p>Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.</p>
--

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

- ☒ Assignee
 ☐ Legal Representative under 35 U.S.C. 117
 ☐ Joint Inventor
- ☐ Person to whom the inventor is obligated to assign.
 ☐ Person who shows sufficient proprietary interest

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name Dow AgroSciences LLC

Mailing Address Information For Applicant:

Address 1 9330 Zionsville Road

Address 2

City Indianapolis

State/Province

IN

Country US

Postal Code

46268

Phone Number

Fax Number

Email Address

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Prefix	Given Name	Middle Name	Family Name	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		State/Province	
Country ⁱ		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Signature	/yonghao hou/	Date (YYYY-MM-DD)	2014-12-02
First Name	Yonghao	Last Name	Hou
Registration Number		61391	

Additional Signature may be generated within this form by selecting the Add button.

This collection of information is required by 37 CFR 1.76. 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 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet 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.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

SCORE Placeholder Sheet for IFW Content

Application Number: 14491197

Document Date: 12/02/2014

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

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Sequence Listing was accepted.
See attached Validation Report.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Anjum, Durreshwar (CGI Federal)
Timestamp: [year=2014; month=12; day=2; hr=14; min=59; sec=15; ms=357;]
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Application No: 14491197 Version No: 2.0

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 245 250 255

Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe Lys Leu Pro
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Val Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp
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Thr Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val
115 120 125

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145 150 155 160

Ala Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln
165 170 175

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180 185 190

Pro Leu Arg Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser
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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
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 35 40 45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
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Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
 65 70 75 80

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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
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 Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
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Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65 70 75 80

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 14/491,197				
APPLICATION AS FILED - PART I										
(Column 1)		(Column 2)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY				
FOR	NUMBER FILED	NUMBER EXTRA	RATE(\$)	FEE(\$)		RATE(\$)	FEE(\$)			
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280			
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	600			
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720			
TOTAL CLAIMS (37 CFR 1.16(i))	26	minus 20 = 6				x 80 =	480			
INDEPENDENT CLAIMS (37 CFR 1.16(h))	4	minus 3 = 1				x 420 =	420			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 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).						400			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00			
			TOTAL			TOTAL	2900			
* If the difference in column 1 is less than zero, enter "0" in column 2.										
APPLICATION AS AMENDED - PART II										
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY		
AMENDMENT A		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x	=		x	=
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x	=		x	=
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))									
					TOTAL ADD'L FEE				TOTAL ADD'L 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 found in the appropriate box in column 1.										



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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

83067

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204

NOTICE



Date Mailed: 12/09/2014

INFORMATIONAL NOTICE TO APPLICANT

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

The item(s) indicated below are also required and should be submitted with any reply to this notice to avoid further processing delays.

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
Pon Samuel Jayakumar



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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO.	TOT CLAIMS	IND CLAIMS
14/491,197	09/19/2014	1638	3040	DAS-P0129-04-US-E	26	4

CONFIRMATION NO. 3336

83067

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204

UPDATED FILING RECEIPT



0C000000072162390

Date Mailed: 12/09/2014

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Terry R. Wright, Carmel, IN;
Justin M. Lira, Zionsville, IN;
Terence Anthony Walsh, Zionsville, IN;
Donald Merlo, Carmel, IN;
Pon Samuel Jayakumar, Carmel, IN;
Gaofeng Lin, Zionsville, IN;

Applicant(s)

Dow AgroSciences LLC, Indianapolis, IN

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 13/647,081 10/08/2012 PAT 8916752

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 09/24/2014

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/491,197**

Projected Publication Date: 03/19/2015

Non-Publication Request: No

Early Publication Request: No

page 1 of 3

Title

NOVEL HERBICIDE RESISTANCE GENES

Preliminary Class

800

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

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Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

IMPROPER CFR REQUEST

83067
Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204



Date Mailed: 12/09/2014

RESPONSE TO REQUEST FOR CORRECTED FILING RECEIPT

Power of Attorney, Claims, Fees, System Limitations, and Miscellaneous

In response to your request for a corrected Filing Receipt, the Office is unable to comply with your request because:

- The ADS submitted on 12/02/2014 was not properly marked up to show the desired changes. For information being changed relative to the information already of record, additions must be shown with underlining, and deletions must be shown with strike-through or brackets. See 37 CFR 1.76(c)(2)

/lvongxay/

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
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Dec 09, 2014 05:23:29 AM

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Indianapolis, IN 46204
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Application	Document	Mailroom Date	Attorney Docket No.
14491197	M327	12/09/2014	DAS-P0129-04-US-E
	APP.FILE.REC	12/09/2014	DAS-P0129-04-US-E
	M327	12/09/2014	DAS-P0129-04-US-E

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		
<p>The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.</p> <p>This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.</p>			

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Terry	R.	Wright		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Carmel	State/Province	IN	Country of Residenceⁱ	US
Mailing Address of Inventor:					
Address 1		14162 Charity Chase Circle			
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46074	Countryⁱ	US		
Inventor 2					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Justin	M.	Lira		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Zionsville	State/Province	IN	Country of Residenceⁱ	US
Mailing Address of Inventor:					
Address 1		8974 Windpointe Pass			
Address 2					
City	Zionsville	State/Province	IN		
Postal Code	46077	Countryⁱ	US		
Inventor 3					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Terence	Anthony	Walsh		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		DAS-P0129-04-US-E	
		Application Number			
Title of Invention		NOVEL HERBICIDE RESISTANCE GENES			
City	Zionsville	State/Province	IN	Country of Residence ⁱ	US
Mailing Address of Inventor:					
Address 1		11638 Promontory Trail			
Address 2					
City	Zionsville	State/Province	IN		
Postal Code	46077	Country ⁱ	US		
Inventor 4					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Donald		Merlo		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Carmel	State/Province	IN	Country of Residence ⁱ	US
Mailing Address of Inventor:					
Address 1		11845 Durbin Drive			
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46032	Country ⁱ	US		
Inventor 5					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	-Por Samuet - Jayakumar	P.	Jayakumar Samuel		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Carmel	State/Province	IN	Country of Residence ⁱ	US
Mailing Address of Inventor:					
Address 1		3336 Kilkenny Circle			
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46032	Country ⁱ	US		
Inventor 6					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Gaofeng		Lin		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Zionsville	State/Province	IN	Country of Residence ⁱ	US

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E	
		Application Number		
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES			
Mailing Address of Inventor:				
Address 1	981 Franklin Trace			
Address 2				
City	Zionsville	State/Province	IN	
Postal Code	46077	Country ⁱ	US	
All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.				<input type="button" value="Add"/>

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).			
☐ An Address is being provided for the correspondence information of this application.			
Customer Number	83067		
Email Address	intear@faegrebd.com	<input type="button" value="Add Email"/>	<input type="button" value="Remove Email"/>

Application Information:

Title of the Invention	NOVEL HERBICIDE RESISTANCE GENES		
Attorney Docket Number	DAS-P0129-04-US-E	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	3	Suggested Figure for Publication (if any)	

Filing By Reference :

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)
Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing. ☐

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	☒ Customer Number	☐ US Patent Practitioner	☐ Limited Recognition (37 CFR 11.9)
Customer Number	83067		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

When referring to the current application, please leave the application number blank.

Prior Application Status	Pending		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
	Continuation of	13647081	2012-10-08		
Prior Application Status	Pending		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
13647081	Continuation of	12091896	2008-11-03		
Prior Application Status	Patented		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12091896	a 371 of international	PCTUS2006042133	2006-10-27	8283522	2012-10-09
Prior Application Status	Expired		Remove		
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)		
PCTUS2006042133	Claims benefit of provisional	60731044	2005-10-28		
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.					

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)ⁱ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	<div>Remove</div> Access Code ⁱ (if applicable)
Additional Foreign Priority Data may be generated within this form by selecting the Add button.			

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

<p>This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.</p> <p>☐ NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.</p>
--

Authorization to Permit Access:

☐ Authorization to Permit Access to the Instant Application by the Participating Offices
<p>If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.</p> <p>In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.</p> <p>In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.</p>

Applicant Information:

<p>Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.</p>
--

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

- ☒ Assignee
 ☐ Legal Representative under 35 U.S.C. 117
 ☐ Joint Inventor
- ☐ Person to whom the inventor is obligated to assign.
 ☐ Person who shows sufficient proprietary interest

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name Dow AgroSciences LLC

Mailing Address Information For Applicant:

Address 1 9330 Zionsville Road

Address 2

City Indianapolis

State/Province

IN

Country US

Postal Code

46268

Phone Number

Fax Number

Email Address

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Prefix	Given Name	Middle Name	Family Name	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		State/Province	
Country ⁱ		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Signature	/yonghao hou/		Date (YYYY-MM-DD)	2014-12-02
First Name	Yonghao	Last Name	Hou	Registration Number
61391				

Additional Signature may be generated within this form by selecting the Add button.

This collection of information is required by 37 CFR 1.76. 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 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet 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.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt	
EFS ID:	21241159
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	83067
Filer:	Yonghao Hou
Filer Authorized By:	
Attorney Docket Number:	DAS-P0129-04-US-E
Receipt Date:	19-JAN-2015
Filing Date:	19-SEP-2014
Time Stamp:	12:25:06
Application Type:	Utility under 35 USC 111(a)

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	Application Data Sheet	DAS-P0129-04-ADSREV12-2-14.pdf	128021 148386d51d131b990e1ea08beef742691eb b9736	no	8

Warnings:

Information:

This is not an USPTO supplied ADS fillable form

Total Files Size (in bytes):

128021

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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.



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

PUBLICATION NOTICE

83067
Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204



OC000000074684300

Title:NOVEL HERBICIDE RESISTANCE GENES

Publication No.US-2015-0080218-A1

Publication Date:03/19/2015

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publicly available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently <http://www.uspto.gov/patft/>.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently <http://pair.uspto.gov/>. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Management, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

To: inteas@faegrebd.com,cynthia.payson@faegrebd.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 83067

Mar 20, 2015 05:25:27 AM

Dear PAIR Customer:

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 83067 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:

The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
14491197	NTC.PUB	03/19/2015	DAS-P0129-04-US-E

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

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 TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:



Practitioners associated with Customer Number:

107540

OR

Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number

Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:



The address associated with Customer Number:

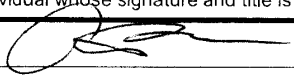
107540

OR

☐	Firm or Individual Name			
	Address			
	City	State	Zip	
	Country			
	Telephone	Email		

Assignee Name and Address: Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268-1054**A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be Filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of The practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.****SIGNATURE of Assignee of Record**

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature		Date	June 21, 2013
Name	Andrea T. Borucki	Telephone	(317) 337-4846
Title	General Patent Counsel		

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.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Wright et al. / Dow AgroSciences LLC
Application No./Patent No.: 14/491,197 Filed/Issue Date: Filed: September 19, 2014
Titled: NOVEL HERBICIDE RESISTANCE GENES
Dow AgroSciences LLC, a Corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.
2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):
- ☐ The extent (by percentage) of its ownership interest is ____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.
- ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (e.g., bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

- 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.
- 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.

[Page 1 of 2]

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.

STATEMENT UNDER 37 CFR 3.73(c)

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.

4. 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.

5. 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.

6. 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(s).

☐ As required by 37 CFR 3.73(c)(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.

/Joshua M. Lee/

Signature

Joshua M. Lee

Printed or Typed Name

February 9, 2016

Date

71650

Title or Registration Number

[Page 2 of 2]

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt	
EFS ID:	24824585
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	83067
Filer:	Joshua M. Lee
Filer Authorized By:	
Attorney Docket Number:	DAS-P0129-04-US-E
Receipt Date:	09-FEB-2016
Filing Date:	19-SEP-2014
Time Stamp:	14:55:10
Application Type:	Utility under 35 USC 111(a)

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	Power of Attorney	Dow_Agrosciences_POA_3_73_c.pdf	270258 467ee24f10462b3468f4a38ba6e0a67253d4470b	no	2

Warnings:

Information:

2	Assignee showing of ownership per 37 CFR 3.73	373c_245772.pdf	117905 9d20cdf3fb83d6e15357d74d6d06c17b408926a1	no	3
Warnings:					
Information:					
Total Files Size (in bytes):			388163		
<p>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.</p> <p><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.</p> <p><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.</p> <p><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.</p>					



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

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

POA ACCEPTANCE LETTER

107540
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204



Date Mailed: 02/22/2016

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 02/09/2016.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/fstephanos/

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Feb 22, 2016 05:29:55 AM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:

The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
14491197	N570	02/22/2016	DAS-P0129-04-US-E

To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO.	TOT CLAIMS	IND CLAIMS
14/491,197	09/19/2014	1638	3040	DAS-P0129-04-US-E	26	4

CONFIRMATION NO. 3336

83067

Faegre Baker Daniels LLP- Dow AgroSciences
300 North Meridian Street, Suite 2700
Indianapolis, IN 46204

UPDATED FILING RECEIPT



0000000072162390

Date Mailed: 12/09/2014

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Terry R. Wright, Carmel, IN;
Justin M. Lira, Zionsville, IN;
Terence Anthony Walsh, ~~Zionsville~~, IN; Carmel
Donald Merlo, Carmel, IN;
~~Pon Samuel Jayakumar~~, Carmel, IN; Jayakumar Pon Samuel
Gaofeng Lin, Zionsville, IN;

Applicant(s)

Dow AgroSciences LLC, Indianapolis, IN

which is a CON of 12/091,896 11/03/2008 PAT 8283522
which is a 371 of PCT/US06/42133 10/27/2006
which claims benefit of 60/731,044 10/28/2005

Power of Attorney: ~~None~~ The patent Practitioners associated with customer no. 107540.

Domestic Priority data as claimed by applicant

This application is a CON of 13/647,081 10/08/2012 PAT 8916752

Foreign Applications for which priority is claimed (You may be eligible to benefit from the Patent Prosecution Highway program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 09/24/2014

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/491,197**

Projected Publication Date: 03/19/2015

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL HERBICIDE RESISTANCE GENES

Preliminary Class

800

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)		Attorney Docket Number	DAS-P0129-04-US-E
		First Named Inventor	Terry R. Wright
		COMPLETE IF KNOWN	
		Application Number	
		Filing Date	
		Art Unit	
☐ Declaration Submitted With Initial Filing OR ☐ Declaration Submitted After Initial Filing (surcharge (37 CFR 1.16(f)) required)		Examiner Name	

I hereby declare that: (1) Each inventor's residence, mailing address, and citizenship are as stated below next to their name; and (2) I believe the inventor(s) named below to be the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention titled:

NOVEL HERBICIDE RESISTANCE GENES

(Title of the Invention)

the application of which was made or was authorized to be made by me and

☒ is attached hereto

OR

☐ was filed on (MM/DD/YYYY) _____ as United States Application Number or PCT International Application Number _____ and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment specifically 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.

Authorization To Permit Access To Application by Participating Offices

☐ If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the above-identified patent application is filed access to the above-identified patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the above-identified patent application is filed to have access to the above-identified patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the above-identified patent application with respect to: 1) the above-identified patent application-as-filed; 2) any foreign application to which the above-identified patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the above-identified patent application; and 3) any U.S. application-as-filed from which benefit is sought in the above-identified patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing the Authorization to Permit Access to Application by Participating Offices.

[Page 1 of 3]

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. 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 21 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.

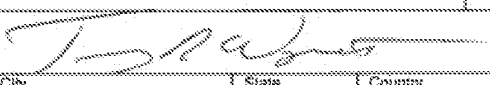
DECLARATION — Utility or Design Patent Application

Claim of Foreign Priority Benefits

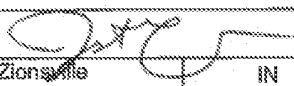
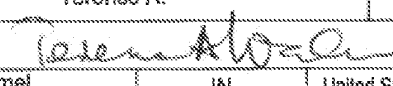
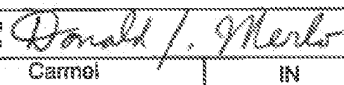
I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

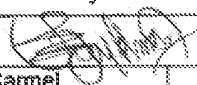
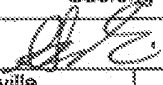
☐ Additional foreign application number(s) are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

DECLARATION — Utility or Design Patent Application			
Direct all correspondence to:		☒ The address associated with Customer Number: 88067	OR ☐ Correspondence address below
Name			
Address			
City		State	ZIP
Country	Telephone	Email	
<p style="text-align: center;">WARNING:</p> <p>Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.</p> <p>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.</p> <p>I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) year, or both.</p>			
NAME OF SOLE OR FIRST INVENTOR:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Terry R.		Wright	
Inventor's Signature		Date	
		7-31-2014	
Residence: City	State	Country	Citizenship
Carmel	IN	United States of America	US
Mailing Address: 14162 Charity Chase Circle			
City	State	Zip	Country
Carmel	IN	46074	United States of America
☒ Additional inventors or a legal representative are being named on the <u>2</u> supplemental sheet(s) PTO/SB/02A or 02L/R attached hereto.			

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION		ADDITIONAL INVENTOR(S) Supplemental Sheet	
		Page 1 of 2	
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Justin M.		Lira	
Inventor's Signature 		Date <u>Aug 6, 2014</u>	
Zionsville	IN	United States of America	US
Residence: City	State	Country	Citizenship
8974 Windpointe Pass			
Mailing Address:			
Zionsville	IN	46077	United States of America
City	State	Zip	Country
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Terence A.		Walsh	
Inventor's Signature 		Date <u>August 6, 2014</u>	
Carmel	IN	United States of America	US
Residence: City	State	Country	Citizenship
Mailing Address: 12443 Branford Street			
Carmel	IN	46032	United States of America
City	State	Zip	Country
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor	
Given Name (first and middle (if any))		Family Name or Surname	
Donald J.		Merlo	
Inventor's Signature 		Date <u>July 31, 2014</u>	
Carmel	IN	United States of America	US
Residence: City	State	Country	Citizenship
Mailing Address: 11845 Durbin Drive			
Carmel	IN	46032	United States of America
City	State	Zip	Country

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION				ADDITIONAL INVENTOR(S) Supplemental Sheet	
				Page <u>2</u> of <u>2</u>	
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Jayakumar Pon			Samuel		
Inventor's Signature 			Date <u>08/18/2014</u>		
Residence: City Carmel	State IN	Country United States of America	Citizenship US		
Mailing Address: 3336 Kilkenny Circle					
City Carmel	State IN	Zip 46032	Country United States of America		
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Gaofeng			Lin		
Inventor's Signature 			Date <u>August 10, 2014</u>		
Residence: City Zionsville	State IN	Country United States of America	Citizenship US		
Mailing Address: 981 Franklin Trace					
City Zionsville	State IN	Zip 46077	Country United States of America		
Name of Additional Joint Inventor, if any:			☐ A petition has been filed for this unsigned inventor		
Given Name (first and middle (if any))			Family Name or Surname		
Inventor's Signature			Date		
Residence: City	State	Country	Citizenship		
Mailing Address:					
City	State	Zip	Country		

Electronic Patent Application Fee Transmittal				
Application Number:		14491197		
Filing Date:		19-Sep-2014		
Title of Invention:		NOVEL HERBICIDE RESISTANCE GENES		
First Named Inventor/Applicant Name:		Terry R. Wright		
Filer:		Elizabeth Lehr Stetzer/Stephanie Switzer		
Attorney Docket Number:		DAS-P0129-04-US-E		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Pet. Delay Sub or Restore Priority-Claim	1454	1	1700	1700
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1700

Electronic Acknowledgement Receipt	
EFS ID:	26690266
Application Number:	14491197
International Application Number:	
Confirmation Number:	3336
Title of Invention:	NOVEL HERBICIDE RESISTANCE GENES
First Named Inventor/Applicant Name:	Terry R. Wright
Customer Number:	107540
Filer:	Elizabeth Lehr Stetzer/Stephanie Switzer
Filer Authorized By:	Elizabeth Lehr Stetzer
Attorney Docket Number:	DAS-P0129-04-US-E
Receipt Date:	19-AUG-2016
Filing Date:	19-SEP-2014
Time Stamp:	12:14:04
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 1700
RAM confirmation Number	081916INTEFSW00009986100435
Deposit Account	100435
Authorized User	Stephanie Switzer
<p>The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:</p> <p>37 CFR 1.16 (National application filing, search, and examination fees)</p> <p>37 CFR 1.17 (Patent application and reexamination processing fees)</p>	

37 CFR 1.19 (Document supply fees)					
37 CFR 1.20 (Post Issuance fees)					
37 CFR 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	Transmittal Letter	Transmittal_8-19-16.pdf	12347	no	3
			99f69f9a3e8c25d9598851d5246b4c2fae1f237d		
Warnings:					
Information:					
2	Miscellaneous Incoming Letter	Petition_8-19-16.pdf	10196	no	2
			644806431cfa2c7d978b986eb8354649fd0cce6c		
Warnings:					
Information:					
3	Application Data Sheet	UpdatedADS_8-19-16.pdf	583247	no	8
			4b9308b7b56738dfefdf1488befbb4c98a46a3a		
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
4	Request for Corrected Filing Receipt	Corrected_Filing_Receipt.pdf	1085141	no	3
			5c5f54db8408264754999a05c23edd2877f76e8		
Warnings:					
Information:					
5	Oath or Declaration filed	14491197Decl.pdf	365506	no	5
			ab2dafb73ecac416e62d17021bf7de56f471fbd6		
Warnings:					
Information:					
6	Fee Worksheet (SB06)	fee-info.pdf	30396	no	2
			4cdeae693a2b62cbcb2ff8e5f4d51dd2219fed7		
Warnings:					

Information:	
Total Files Size (in bytes):	2086833
<p>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.</p> <p><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.</p> <p><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.</p> <p><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.</p>	

BARNES & THORNBURG LLP

11 South Meridian Street
Indianapolis, Indiana 46204
(317) 236-1313
(317) 231-7433 Fax

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>Customer No.</i>	107540	}
		}
<i>Group:</i>	1638	}
		}
<i>Confirmation No.:</i>	3336	}
		}
<i>Application No.:</i>	14/491,197	}
		}
<i>Invention:</i>	Novel Herbicide Resistance Genes	}
		}
<i>Inventor:</i>	Terry Wright et al.	}
		}
<i>Filed:</i>	September 19, 2014	}
		}
<i>Attorney</i>		}
<i>Docket:</i>	14764-245772	}
		}
<i>Examiner:</i>	Unknown	}

**FILED ELECTRONICALLY:
August 19, 2016**

**NEW APPLICATION DATA SHEET AND
REQUEST FOR CORRECTED FILING RECEIPT**

Commissioner For Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

Applicant submits herewith a new Application Data Sheet (Form AIA/14) and Request for Corrected Filing Receipt. The new Application Data Sheet submitted herewith reflects the correction of the following typographical errors in the Application Data Sheet as originally filed on September 19, 2014:

- (1) A typographical error in the name of Inventor 5 has been corrected from “Pon Samuel Jayakumar” to Jayakumar Pon Samuel”;
- (2) The mailing address of Inventor 3 has been corrected; and
- (3) A typographical error in the “Domestic Benefit/National Stage Information” section has been corrected.

Applicant further submits herewith a PETITION UNDER 37 C.F.R. § 1.78 TO ACCEPT AN UNINTENTIONALLY DELAYED CLAIM OF PRIORITY. The Petition indicates that the complete and correct priority claim was provided in the specification as filed on September 19, 2014. The priority claim in the Application Data Sheet as filed contained a typographical error which has been corrected on the new Application Data Sheet submitted herewith.

Applicant respectfully requests that a Corrected Filing Receipt be issued showing the corrected spelling of the name of Inventor 5, the corrected address of Inventor 3, and corrected priority claim data as indicated on the new Application Data Sheet submitted herewith. Applicant additionally requests that the Corrected Filing Receipt reflect the change in Power of Attorney to the practitioners with Customer Number 107540. A Power of Attorney appointing the practitioners associated with Customer Number 107540 was submitted by Applicant on February 9, 2016, and the Power of Attorney was accepted by the USPTO on February 22, 2016. A copy of the filing receipt for the above-captioned patent application is attached with the requested changes noted in writing. Applicant also requests that the corrected filing receipt be sent to the undersigned attorney.

In response to the ITEMS REQUIRED TO AVOID PROCESSING DELAYS, Applicant submits herewith a inventor’s Oath or Declaration executed by Inventors Terry R. Wright, Justin M. Lira, Terence A Walsh, Donald J. Merlo, Jayakumar Pon Samuel, and Gaofeng Lin. Applicant notes that the “Late Filing Fee for Oath or Declaration” of \$140 was previously submitted in the USPTO on December 2, 2014.

Applicant believes that no additional fees other than the Petition fee are required with this submission. However, if any additional fees are required, the Commissioner is hereby authorized to charge any fees as necessary to the Barnes & Thornburg Deposit Account No. 10-0435, with reference to our matter number 14764-245772.

Respectfully submitted,

/Elizabeth Lehr Stetzer/
Elizabeth Lehr Stetzer
Reg. No. 63496

Indianapolis, Indiana
(317) 231-7228

DMS ESTETZER 4271756v1

BARNES & THORNBURG LLP

11 South Meridian Street
Indianapolis, Indiana 46204
(317) 236-1313
(317) 231-7433 Fax

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>Customer No.</i>	107540	}
		}
<i>Group:</i>	1638	}
		}
<i>Confirmation No.:</i>	3336	}
		}
<i>Application No.:</i>	14/491,197	}
		}
<i>Invention:</i>	Novel Herbicide Resistance Genes	}
		}
<i>Inventor:</i>	Terry Wright et al.	}
		}
<i>Filed:</i>	September 19, 2014	}
		}
<i>Attorney</i>		}
<i>Docket:</i>	14764-245772	}
		}
<i>Examiner:</i>	Unknown	}

**FILED ELECTRONICALLY:
August 19, 2016**

PETITION UNDER 37 C.F.R. § 1.78
TO ACCEPT AN UNINTENTIONALLY DELAYED CLAIM OF PRIORITY

Dear Sirs:

Petitioner respectfully requests the Commissioner to accept an unintentionally delayed claim of priority for the instant application. The specification as filed on September 19, 2014 included the complete and correct priority claim as follows:

This application is a continuation of U.S. patent application Ser. No. 13/647,081, filed on Oct. 8, 2012, which is a continuation of U.S. Pat. No. 8,283,522 with Ser. No. 12/091,896, filed on Nov. 3, 2008, which claims the benefit of PCT

International Application Serial No. PCT/US2006/042133, filed on Oct. 27, 2006, which claims the benefit of U.S. Provisional Application Ser. No. 60/731,044, filed on Oct. 28, 2005.... (See paragraph [0001] of Publication No. US 2015-0080218).

However, the original Application Data Sheet, also filed on September 19, 2014, inadvertently omitted a portion of the priority claim. Thus, the priority claim was correctly listed in the Application specification itself as evidence of Applicant's intent to claim priority.

Submitted herewith is a new Application Data Sheet incorporating reference to all previously-filed applications as listed in the specification as filed. The entire delay between the date such claim was due and the date of submission of this petition was unintentional.

The Office is hereby authorized to charge the required petition fee for a large entity as set forth in 37 C.F.R. § 1.17(m) to the Barnes & Thornburg Deposit Account No. 10-0435, with reference to our matter number 14764-245772.

It is believed that the above-authorized fee is correct; however, the Commissioner is authorized to charge any additional fees which may be required to the Barnes & Thornburg Deposit Account No. 10-0435, with reference to our matter number 14764-245772.

If any matter needs further clarification or supplemental support, the Petitions Attorney is invited to contact the undersigned via telephone so the matter may be promptly addressed.

Respectfully submitted,

/Elizabeth Lehr Stetzer/
Elizabeth Lehr Stetzer
Reg. No. 63496

Indianapolis, Indiana
Phone No.: (317) 231-7228

DMS ESTETZER 4273436v1

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		
<p>The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.</p> <p>This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.</p>			

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove				
Legal Name				
Prefix	Given Name	Middle Name	Family Name	Suffix
	Terry	R.	Wright	
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service				
City	Carmel	State/Province	IN	Country of Residence US
Mailing Address of Inventor:				
Address 1		14162 Charity Chase Circle		
Address 2				
City	Carmel	State/Province	IN	
Postal Code	46074	Country	US	
Inventor 2 Remove				
Legal Name				
Prefix	Given Name	Middle Name	Family Name	Suffix
	Justin	M.	Lira	
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service				
City	Zionsville	State/Province	IN	Country of Residence US
Mailing Address of Inventor:				
Address 1		8974 Windpointe Pass		
Address 2				
City	Zionsville	State/Province	IN	
Postal Code	46077	Country	US	
Inventor 3 Remove				
Legal Name				
Prefix	Given Name	Middle Name	Family Name	Suffix
	Terence	Anthony	Walsh	
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service				

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		DAS-P0129-04-US-E	
		Application Number			
Title of Invention		NOVEL HERBICIDE RESISTANCE GENES			

City	Zionsville	State/Province	IN	Country of Residence	US
------	------------	----------------	----	----------------------	----

Mailing Address of Inventor:

Address 1	11030 Promontory Trail 12443 Branford Street				
Address 2					
City	Zionsville	Carmel	State/Province	IN	
Postal Code	46077	46032	Country	US	
Remove					

Inventor 4

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Donald		Merlo	

Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service

City	Carmel	State/Province	IN	Country of Residence	US
------	--------	----------------	----	----------------------	----

Mailing Address of Inventor:

Address 1	11845 Durbin Drive				
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46032	Country	US		
Remove					

Inventor 5

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Pon Samuel Jayakumar	Pon	Jayakumar Samuel	

Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service

City	Carmel	State/Province	IN	Country of Residence	US
------	--------	----------------	----	----------------------	----

Mailing Address of Inventor:

Address 1	3336 Kilkenny Circle				
Address 2					
City	Carmel	State/Province	IN		
Postal Code	46032	Country	US		
Remove					

Inventor 6

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Gaofeng		Lin	I

Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service

City	Zionsville	State/Province	IN	Country of Residence	US
------	------------	----------------	----	----------------------	----

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		DAS-P0129-04-US-E	
		Application Number			
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES				
Mailing Address of Inventor:					
Address 1	981 Franklin Trace				
Address 2					
City	Zionsville	State/Province	IN		
Postal Code	46077	Country	US		
All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button. <input type="button" value="Add"/>					

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ An Address is being provided for the correspondence information of this application.

Customer Number	00007 107540
Email Address	info@faegrebbd.com INdocket@btlaw.com <input type="button" value="Add Email"/> <input type="button" value="Remove Email"/>

Application Information:

Title of the Invention	NOVEL HERBICIDE RESISTANCE GENES		
Attorney Docket Number	DAS-P0129-04-US-E	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	3	Suggested Figure for Publication (if any)	
Filing By Reference :			
Only complete this section when filing an application by reference under 35 U.S.C. 411(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").			
For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).			
Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country	

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)
☐ Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.			
Please Select One:	☒ Customer Number	☐ US Patent Practitioner	☐ Limited Recognition (37 CFR 11.9)
Customer Number	00067 107540		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78. When referring to the current application, please leave the application number blank.					
Prior Application Status		Pending		Remove	
Application Number		Continuity Type		Prior Application Number	Filing Date (YYYY-MM-DD)
		Continuation of		13647081	2012-10-08
Prior Application Status		<u>Patented</u>		Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
<u>13647081</u>	<u>Continuation of</u>	<u>12091896</u>	<u>2008-11-03</u>	<u>8283522</u>	<u>2012-10-09</u>
Prior Application Status		Patented <u>Expired</u>		Remove	
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
12091896	a 371 of International	PCTUS2006042133	2006-10-27	8283522	2012-10-09
Prior Application Status		Expired		Remove	
Application Number		Continuity Type		Prior Application Number	Filing Date (YYYY-MM-DD)
PCTUS2006042133		Claims benefit of provisional		60731044	2005-10-28
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.					

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) ¹ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).	

EFS Web 2.2.11

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Application Number	Country ¹	Filing Date (YYYY-MM-DD)	Access Code ¹ (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

☐ NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization to Permit Access:

☐ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	DAS-P0129-04-US-E
	Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES	

Applicant 1			
If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.			
<input type="button" value="Clear"/>			
☒ Assignee		☐ Legal Representative under 35 U.S.C. 117	
☐ Person to whom the inventor is obligated to assign.		☐ Joint Inventor	
☐ Person who shows sufficient proprietary interest			
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:			
Name of the Deceased or Legally Incapacitated Inventor : <input type="text"/>			
If the Applicant is an Organization check here. ☒			
Organization Name		Dow AgroSciences LLC	
Mailing Address Information For Applicant:			
Address 1		9330 Zionsville Road	
Address 2			
City		Indianapolis	State/Province IN
Country	US	Postal Code	46268
Phone Number		Fax Number	
Email Address			
Additional Applicant Data may be generated within this form by selecting the Add button.			

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.	
Assignee 1	
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.	
If the Assignee or Non-Applicant Assignee is an Organization check here. ☐	

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	DAS-P0129-04-US-E
		Application Number	
Title of Invention	NOVEL HERBICIDE RESISTANCE GENES		

Prefix	Given Name	Middle Name	Family Name	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:				
Address 1				
Address 2				
City		State/Province		
Country ¹		Postal Code		
Phone Number		Fax Number		
Email Address				
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.				

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.				
Signature	/Elizabeth Stetzer/		Date (YYYY-MM-DD)	2016-08-19
First Name	Elizabeth	Last Name	Stetzer	Registration Number
63496				
Additional Signature may be generated within this form by selecting the Add button.				

This collection of information is required by 37 CFR 1.76. 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 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet 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.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Document code: WFEE

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Alexandria, Virginia 22313-1450
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

107540
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

37 CFR 1.48(f)
ACKNOWLEDGEMENT LETTER



Date Mailed: 08/30/2016

NOTICE OF ACCEPTANCE OF REQUEST UNDER 37 CFR 1.48(f)

This is in response to the applicant's request under 37 CFR 1.48(f) submitted on 08/19/2016.

The request under 37 CFR 1.48(f) to correct the inventorship, to correct or update the name of an inventor, or to correct the order of names of joint inventors is accepted.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/byemanc/



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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO.	TOT CLAIMS	IND CLAIMS
14/491,197	09/19/2014	1663	3040	DAS-P0129-04-US-E	26	4

CONFIRMATION NO. 3336
UPDATED FILING RECEIPT

107540
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204



0000000085419591

Date Mailed: 08/30/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Terry R. Wright, Carmel, IN;
Justin M. Lira, Zionsville, IN;
Terence Anthony Walsh, Zionsville, IN;
Donald Merlo, Carmel, IN;
Jayakumar Pon Samuel, Carmel, IN;
Gaofeng Lin, Zionsville, IN;

Applicant(s)

Dow AgroSciences LLC, Indianapolis, IN;

Power of Attorney: The patent practitioners associated with Customer Number 107540

Domestic Priority data as claimed by applicant

This application is a CON of 13/647,081 10/08/2012 PAT 8916752
which is a CON of 12/091,896 11/03/2008 PAT 8283522
which is a 371 of PCT/US06/42133 10/27/2006
which claims benefit of 60/731,044 10/28/2005

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: No

Permission to Access Search Results: No

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted:

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/491,197**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL HERBICIDE RESISTANCE GENES

Preliminary Class

800

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

page 2 of 4

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15**

GRANTED

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This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Aug 30, 2016 05:26:27 AM

Dear PAIR Customer:

Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 107540 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	R48.REQ.G	08/30/2016	DAS-P0129-04-US-E
	APP.FILE.REC	08/30/2016	DAS-P0129-04-US-E

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E

CONFIRMATION NO. 3336

107540
Barnes & Thornburg LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

**PUB REQUEST ACCEPTANCE
LETTER**



Date Mailed: 10/07/2016

NOTICE OF ACCEPTANCE OF PUBLICATION REQUEST

The request for voluntary publication, amended publication, early publication, redacted publication, republication, corrected publication or revised publication has been received for this application. The request, including payment of any necessary fee(s), is in compliance with 37 CFR 1.215, 1.217, 1.219 or 1.221.

The projected publication date is 01/12/2017.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/dterry/

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Oct 07, 2016 05:28:00 AM

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11 South Meridian Street
Indianapolis, IN 46204
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	M327	10/07/2016	DAS-P0129-04-US-E

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Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

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MAILED

OCT 06 2016

INTERNATIONAL PATENT LEGAL ADM.

107540
BARNES & THORNBURG LLP (Dow)
11 South Meridian Street
Indianapolis, IN 46204

In re Application of :
DOW AGROSCIENCES LLC :
Application No.: 14/491,197 :
Filing Date: September 19, 2014 :
Attorney Docket No.: DAS-P0129-04-US-E :
Re.: NOVEL HERBICIDE RESISTANCE GENES :

DECISION ON PETITION
UNDER 37 CFR 1.78(c)
AND 1.78(e)

This is a decision on the petition under 37 CFR 1.78(c) and (e), filed August 19, 2016, to accept an unintentionally delayed claim under 35 U.S.C. 119(e) and 120 for the benefit of priority to one or more prior-filed provisional and nonprovisional/international applications.

Under 37 CFR 1.78(c) and (e), a petition to accept an unintentionally delayed claim under 35 U.S.C. 119(e) and 120 for the benefit of a prior-filed application must be accompanied by:

- (i) the reference required by 35 U.S.C. 119(e) and 120 and 37 CFR 1.78(a)(3) and 1.78(d)(2) to the prior-filed applications, unless previously submitted;
- (ii) the petition fee set forth in 37 CFR 1.17(m); and
- (iii) a statement that the entire delay between the date the claim was due under 37 CFR 1.78(a)(4) and (d)(3) and the date the claim was filed was unintentional. The Director may require additional information where there is a question whether the delay was unintentional.

With regard to item (i), a proper reference to the prior-filed applications has been included in an application data sheet as required by 37 CFR 1.78(a)(3) and (d)(2).

With regard to item (ii), the petition fee set forth in 37 CFR 1.17(m) has been submitted.

With regard to item (iii), the statement of unintentional delay contained in the petition differs slightly from the language contained in 37 CFR 1.78(c)(3) and (e)(3) and is hereby construed as a statement that the entire delay between the date the claim was due under 1.78(a)(4) and (d)(3) and the date the claim was filed was unintentional. If this interpretation is incorrect, applicant is required to immediately notify the Office. As construed, the provided statement of unintentional delay is acceptable.

For the reasons above, the petition under 37 CFR 1.78(c) and (e) is **GRANTED**.

Applicant is advised that the inclusion of a prior-filed application on any filing receipt should not be construed as meaning that the application is necessarily entitled to the benefit of the prior-filed application. In order for an application to be entitled to the benefit of the prior-filed application, all other requirements under 35 U.S.C. 119(e) and 120 and 37 CFR 1.78 must be met. The examiner will, in due course, determine whether this application is entitled to the benefit of an earlier filing date.

This matter is being referred to Technology Center Art Unit 1663 for appropriate action, including consideration by the examiner of applicant's entitlement to the benefit of priority under 35 U.S.C. 119(e) and 120 to any prior-filed applications. A corrected filing receipt showing the changes to the priority claims was issued August 30, 2016

/James Thomson/

JAMES THOMSON
Attorney Advisor
International Patent Legal Administration
571-272-3302

To: INDocket@btlaw.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 107540

Oct 12, 2016 05:27:29 AM

Dear PAIR Customer:

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Indianapolis, IN 46204
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	PET.DEC.OIPE	10/06/2016	DAS-P0129-04-US-E

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/491,197	09/19/2014	Terry R. Wright	DAS-P0129-04-US-E	3336
107540	7590	01/12/2017	EXAMINER	
Barnes & Thornburg LLP (Dow)			KRUSE, DAVID H	
11 South Meridian Street			ART UNIT	PAPER NUMBER
Indianapolis, IN 46204			1663	
			NOTIFICATION DATE	DELIVERY MODE
			01/12/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com

Office Action Summary	Application No. 14/491,197	Applicant(s) WRIGHT ET AL.	
	Examiner DAVID H. KRUSE	Art Unit 1663	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☐ Responsive to communication(s) filed on _____.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

- 5) ☒ Claim(s) 1-26 is/are pending in the application.
5a) Of the above claim(s) 10 is/are withdrawn from consideration.
- 6) ☐ Claim(s) _____ is/are allowed.
- 7) ☒ Claim(s) 1-9 and 11-26 is/are rejected.
- 8) ☐ Claim(s) _____ is/are objected to.
- 9) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

- 10) ☒ The specification is objected to by the Examiner.
- 11) ☒ The drawing(s) filed on 9/19/2014 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

- a) ☐ All b) ☐ Some** c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)
Paper No(s)/Mail Date _____.
- 3) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____.
- 4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Information Disclosure Statement

1. The listing of references on pages 134-141 of the specification is not a proper information disclosure statement. 37 CFR 1.98(b) requires a list of all patents, publications, or other information submitted for consideration by the Office, and MPEP § 609.04(a) states, "the list may not be incorporated into the specification but must be submitted in a separate paper." Therefore, unless the references have been cited by the examiner on form PTO-892, they have not been considered.

Specification

2. The disclosure is objected to because of the following informalities: The table on page 105 is not numbered.

Appropriate correction is required.

Claim Objections

3. Claim 10 is objected to under 37 CFR 1.75(c) as being in improper form because a multiple dependent claim should refer to other claims in the alternative. See MPEP § 608.01(n). Accordingly, the claim 10 is not been further treated on the merits.

4. Claims 4 and 19 are objected to because of the following informalities: The phrase "an expression cassette" should read -- the expression cassette -- in referring to claim 1. Appropriate correction is required.

Double Patenting

5. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file

provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based e-Terminal Disclaimer may be filled out completely online using web-screens. An e-Terminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about e-Terminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

6. Claims 1-9 and 11-26 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-14 of U.S. Patent No. 8,283,522. Although the claims at issue are not identical, they are not patentably distinct from each other because the transgenic plant cell of claim 1 of the '522 patent render the expression cassette of instant claim 1 obvious because such an expression cassette would have been required to make said transgenic plant cell. The method of selecting a transgenic plant at instant claim 5 would have been obvious in view of said transgenic plant cell because it is how said transgenic plant cell would have been identified. The method of instant claim 4 substantially overlaps in scope with claim 3 of the '522 patent. The polynucleotide of claims 21-26 encompasses a genus anticipated or rendered obvious by the plant cell of claim 1 of the '522 patent.

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7. Claims 1-9 and 11-26 are rejected on the ground of nonstatutory double

patenting as being unpatentable over claims 1-18 of U.S. Patent No. 8,916,752.

Although the claims at issue are not identical, they are not patentably distinct from each other because the transgenic plant of claim 1 of the '752 patent render the expression cassette of instant claim 1 obvious because such an expression cassette would have been required to make said transgenic plant. The method of selecting a transgenic plant at instant claim 5 would have been obvious in view of said transgenic plant because it is how said transgenic plant would have been identified. The method of instant claim 4 substantially overlaps in scope with claim 18 of the '752 patent. The polynucleotide of claims 21-26 encompasses a genus anticipated or rendered obvious by the plant cell of claim 1 of the '752 patent.

8. Claims 1-9 and 11-26 are rejected on the ground of nonstatutory double

patenting as being unpatentable over claims 1-28 of U.S. Patent No. 8,278,505.

Although the claims at issue are not identical, they are not patentably distinct from each other because the transgenic plant cell or claim 2 of the '505 patent render the expression cassette of instant claim 1 obvious because such an expression cassette would have been required to make said transgenic plant cell. The method of selecting a transgenic plant at instant claim 5 would have been obvious in view of said transgenic plant cell because it is how said transgenic plant cell would have been identified. The method of instant claim 4 substantially overlaps in scope with claim 11 of the '505 patent. The polynucleotide of claims 21-26 encompasses a genus anticipated or rendered obvious by the isolated polynucleotide of claim 1 of the '505 patent.

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9. Claims 4, 6-9 and 11-18 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 7-12 of U.S. Patent No. 9,062,294.

Although the claims at issue are not identical, they are not patentably distinct from each other because the instant claims substantially overlap in scope with said claims of the '294 patent, i.e. using a transgenic plant comprising a transgene encoding a polypeptide of either SEQ ID NO: 2 or SEQ ID NO: 4 to control weeds in a field.

10. Claims 4, 6-9 and 11-18 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 30-37 of copending Application No. 15/184,601. Although the claims at issue are not identical, they are not patentably distinct from each other because the method step(s) of the instantly claimed invention are substantially the same as the method step(s) of the copending application. The only difference being the intended outcome of the method(s).

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Claim Rejections - 35 USC § 101

11. 35 U.S.C. 101 reads as follows:

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

12. Claims 1, 2 and 21-24 are rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. Claim(s) 1, 2 and 21-24 is/are directed to a product of nature. The claim(s) does/do not include additional elements that are sufficient to amount to significantly more than the judicial exception because the

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limitations “promoter that is functional in a plant cell” and “comprises plant codons for expression in said plant cell” do not structurally distinguish the claimed products from a product of nature. Schleinitz *et al* (2004) teach that the *SdpA* gene which encodes instant SEQ ID NO: 2 is present on a plasmid designated pMC1 (see the abstract on page 5357). The designation “expression cassette” merely denotes an intended use of a piece of DNA. Further, bacterial promoters can be functional in a plant plastid, and Maliga (2002) teaches that for expression of a bacterial gene in a plant plastid, any codon optimization has very little effect on protein expression (page 167, right column, last paragraph; page 170, right column, 3rd paragraph).

Claim Rejections - 35 USC § 112

13. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

(a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

14. Claims 21-26 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint

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inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant claims a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity that hybridizes under stringent conditions to SEQ ID NO: 1, 3 or 5.

Applicant describes polynucleotides that encode having aryloxyalkanoate dioxygenase activity in SEQ ID NO: 2 and 4. Applicant further describes "stringent conditions" in very general terms on pages 22-23.

Applicant does not describe the genus of polynucleotides encoding a protein having aryloxyalkanoate dioxygenase activity other than in what said polynucleotides do, hybridize, and not what they are.

Hence, it is unclear that Applicant was in possession of the invention as broadly claimed. See *University of Rochester v. G.D. Searle & Co.*, 68 USPQ2d 1424, 1433 (DC WNY 2003) which teaches knowing the "starting point" is not enough; that is little more than a research plan. The court held that the disclosure of screening assays and general classes of compounds was not adequate to describe compounds having the desired activity: without disclosure of which peptides, polynucleotides, or small organic molecules have the desired characteristic, the claims failed to meet the description requirement of § 112.

15. The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

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16. Claims 8 and 9 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

At claim 8, it is unclear if said claim is limiting the method of claim 4 to use of a pyridyloxy auxin herbicide or merely limiting the selection of pyridyloxy auxin herbicides but a phenoxy auxin herbicide can still be selected in the methods of claim 8 and 9. Hence, the metes and bounds of claims 8 and 9 are unclear.

Claim Rejections - 35 USC § 102

17. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

18. Claim(s) 1, 2 and 21-24 are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Schleinitz *et al* (2004 Applied and Environmental Microbiology 70(9): 5357-5365) taken with the evidence of Maliga (2002 Current Opinion in Plant Biology 5: 164-172).

Schleinitz *et al* (2004) disclose that the SdpA gene which encodes instant SEQ ID NO: 2 and would hybridize under “stringent conditions” with the full complement of instant SEQ ID NO: 1 is present on a plasmid designated pMC1 (see the abstract on page 5357). The designation “expression cassette” merely denotes an intended use of a piece of DNA. Further, bacterial promoters can be functional in a plant plastid, and

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Maliga (2002) teaches that for expression of a bacterial gene in a plant plastid, any codon optimization has very little effect on protein expression (page 167, right column, last paragraph; page 170, right column, 3rd paragraph). Hence, Schleinitz *et al* had previously disclosed the claim limitations.

Claim Rejections - 35 USC § 103

19. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

20. Claims 1-7 and 11-26 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz *et al* (September 2004, Applied and Environmental Microbiology 70(9): 5357-5365), Pallett *et al* (U.S. Patent 7,250,561 B1, § 371(c)(1) date of 15 June 1998) and Xu *et al* (U.S. Patent 6,665,384).

Kaphammer teaches a plant cell comprising a polynucleotide operably linked to a CAMV 35S promoter (Figure 3) that encodes a protein having aryloxyalkanoate dioxygenase activity, resistance to 2,4-dichlorophenoxyacetate (2,4-D) at claim 11, and a plant regenerated therefrom at claim 15. Kaphammer teaches an isolated polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity at SEQ ID NO: 1. Kaphammer teaches transforming a soybean cell at Example 11, columns 21-22. Kaphammer teaches a method of controlling weeds by applying 2,4-D herbicide to a crop field at column 2, lines 26-28. Kaphammer teaches that a transgene

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that confers resistance to glyphosate can also be introduced into the plant cell (column 3, lines 11-6).

Kaphammer does not teach an isolated nucleic acid encoding instant SEQ ID NO: 2 or having the nucleotide sequence of instant SEQ ID NO: 1. Kaphammer does not teach introducing a polynucleotide that confers glufosinate resistance. Kaphammer does not teach using a cassava vein mosaic virus promoter.

Schleinitz *et al* teach an isolated polynucleotide comprising instant SEQ ID NO: 1 at GenBank accession number AY327575 (page 5359, right column, 5th paragraph). The encoded *SdpA* protein has aryloxyalkanoate dioxygenase activity and has greatest activity of degrading S-enantiomers of mecoprop and dichlorprop and has some activity toward 2,4-D (page 5363, left column, 2nd paragraph).

Pallett *et al* teach introducing polynucleotides to a transformed plant cell that renders said plant cell resistant to multiple herbicides including glyphosate (column 1, lines 62-64) and glufosinate (column 2, lines 27-29).

Xu *et al* teach a cassava vein mosaic virus promoter operably linked to a sequence of interest at Figure 3. Xu *et al* teach that genes encoding herbicide resistance can be used as selectable markers (column 7, lines 5-12) and that said sequence of interest can be a selectable marker at claim 27.

It would have been *prima facie* obvious to one of ordinary skill in the art at the time of Applicants' invention to modify the teachings of Kaphammer to introduce a polynucleotide encoding instant SEQ ID NO: 2 as taught by Schleinitz *et al* in addition to a polynucleotide encoding resistance to glyphosate and glufosinate as suggested Pallett

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et al. Given the success of Pallett *et al.*, and Kaphammer one of ordinary skill in the art would have had a reasonable expectation of success. Given dichlorprop was a known herbicide as taught by Schleinitz *et al.* (page 5357, left column, 1st paragraph), one of ordinary skill in the art would have been motivated to introduce said polynucleotide encoding instant SEQ ID NO: 2 into a plant cell for the purposes of making an herbicide tolerant plant from said plant cell. The method of instant claim 5 is essentially the same as a method of controlling weeds wherein the herbicide tolerant plant is selected by applying an herbicide, such as those methods taught by Kaphammer and Pallett *et al.* It would have been obvious to one of ordinary skill in the art at the time of Applicant's invention to substitute functionally similar/equivalent transgenes to produce a phenoxy auxin herbicide resistant plant.

Conclusion

21. No claims are allowed.

22. Claims 8 and 9 appear free of the prior art. The closest art is Crawford *et al.* which teaches degrading triclopyr (column 3, lines 21-22) using a 2,4-D a-ketoglutarate dioxygenase at claims 3 and 6.

23. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799 and direct fax number (571) 273-0799. The examiner can normally be reached on Monday to Friday from 7:30 a.m. to 4:00 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amjad Abraham can be reached at (571) 270-7058. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/
Primary Examiner, Art Unit 1663

Notice of References Cited	Application/Control No. 14/491,197		Applicant(s)/Patent Under Reexamination WRIGHT ET AL.	
	Examiner DAVID H. KRUSE		Art Unit 1663	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-5,608,147 A	03-1997	Kaphammer; Bryan J.	C12N9/0004	435/410
*	B	US-8,278,505 B2	10-2012	Lira; Justin M.	C12N9/0004	435/419
*	C	US-9,062,284 B2	06-2015	Lira; Justin M.	C12N9/0004	1/1
*	D	US-8,916,752 B2	12-2014	Wright; Terry R.	C12N9/0069	435/419
*	E	US-8,283,522 B2	10-2012	Wright; Terry R.	C12N9/0069	435/419
*	F	US-6,664,384 B1	12-2003	Xu; Dongmei	C12N15/8216	435/320.1
*	G	US-7,250,561 B1	07-2007	Pallett; Ken	C12N9/0069	435/320.1
*	H	US-5,656,422 A	08-1997	Crawford; Ronald L.	C12Q1/26	435/174
	I	US-				
	J	US-				
	K	US-				
	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Schleinitz et al September 2004, Applied and Environmental Microbiology 70(9): 5357-5365
	V	Fourgoux-Nicol et al 1999, Plant Molecular Biology 40: 857-872
	W	Maliga 2002, Current Opinion in Plant Biology 5: 164-172
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Engineering the plastid genome of higher plants

Pal Maliga

The plastid genome of higher plants is an attractive target for engineering because it provides readily obtainable high protein levels, the feasibility of expressing multiple proteins from polycistronic mRNAs and gene containment through the lack of pollen transmission. A chloroplast-based expression system that is suitable for the commercial production of recombinant proteins in tobacco leaves has been developed recently. This expression system includes vectors, expression cassettes and site-specific recombinases for the selective elimination of marker genes. Progress in expressing proteins that are biomedically relevant, in engineering metabolic pathways, and in manipulating photosynthesis and agronomic traits is discussed, as are the problems of implementing the technology in crops.

Addresses

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Current Opinion in Plant Biology 2002, 5:164–172

1369-5266/02/\$ —see front matter

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Abbreviations

AAD	aminoglycoside 3'-adenyltransferase
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GFP	green fluorescent protein
MCS	multiple cloning site
NPTII	neomycin phosphotransferase II
PAT	phosphinothricin acetyltransferase
PEP	plastid-encoded plastid RNA polymerase
pPRV	plastid repeat vector
PPT	phosphinothricin
Prrn	rRNA operon promoter
RBS	ribosome binding site
5'-UTR	5'-untranslated mRNA region
T7g10	T7-phage gene 10
TSP	total soluble cellular protein

Introduction

Plastids of higher plants are cellular organelles with circular, double-stranded genomes of 120–160 kilobases in size. The genome of each plastid encodes approximately 120 genes: each cell contains up to 10 000 identical copies of each plastid gene. To obtain a genetically stable plant all genome copies have to be uniformly transformed. Plastid transformation is obtained through the following steps: first, the introduction of transforming DNA that encodes a selectable marker (e.g. an antibiotic resistance gene) by the biolistic process or by polyethylene glycol treatment; second, the integration of the transforming DNA by two homologous recombination events (Figure 1); and third, the gradual elimination of wildtype genome copies during repeated cell divisions on a selective medium [1*].

This review focuses on the production of recombinant proteins in the chloroplasts of higher plants. Most of the

examples discussed utilize tobacco, as this is the only species in which plastid transformation is routinely obtained. Emerging applications for plastid transformation, progress in extending the technology to new crops and the potential for gene containment using plastid transformation are also discussed. Other applications of plastid transformation in higher plants and in *Chlamydomonas*, a unicellular alga, have been reviewed elsewhere [1*,2,3].

Vectors

Plastid transformation vectors utilize spectinomycin and streptomycin resistance as selective markers conferred by the bacterial *aadA* gene, which encodes aminoglycoside 3'-adenyltransferase (AAD). The plastid repeat vector (pPRV) series [4] and the vectors pRB94 and pRB95 [5**] are advanced vectors, in which a chimeric *aadA* is linked to unique restriction sites known as multiple cloning sites (MCS) (Figure 2). The pPRV vectors target insertions in the *trnV-rps12/7* intergenic region, whereas pRB94 and pRB95 target the *trnM-trnG* intergenic region. The shuffling of expression signals and coding regions via unique restriction sites in the MCS facilitates the modular construction of chimeric genes (Figure 3).

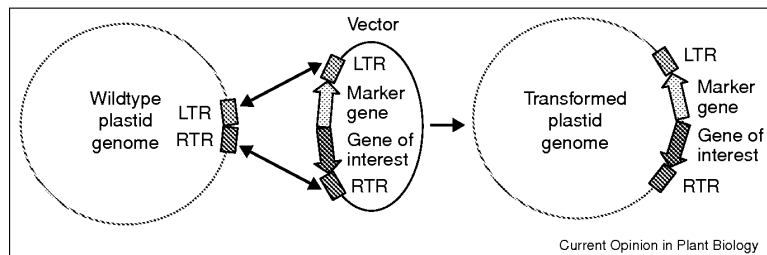
Read-through transcription facilitates the expression of ribosome binding site (RBS)-coding region segments that are inserted at intergenic regions, allowing the protein of interest to be translated from a polycistronic mRNA. This approach has been adopted for the expression of heterologous proteins by taking advantage of unique *XbaI* and *SpeI* restriction sites downstream of *aadA* in the plasmid pZS197 [6]. The RBS-coding region segments were cloned either directly in plasmid pZS197 [7,8] or after inserting *aadA* in the *trnI-trnA* intergenic region of a universal vector [9**,10,11]. Read-through transcription is undesirable if expression of the plastid transgene is regulated transcriptionally [2]. For information on read-through transcription in vectors see Figure 2.

Marker genes

The choice of selective marker gene and selective agent are critical for successful transformation. Selective plastid markers are spectinomycin-streptomycin and kanamycin resistance, conferred by the expression of chimeric *aadA* [6] and *neo* genes [12], respectively. A new positive-selection scheme involves the identification of transplastomic tobacco lines by their resistance to betaine aldehyde conferred by expression of betaine aldehyde dehydrogenase, which is encoded by a plant nuclear gene [13*]. The bacterial *bar* gene, encoding phosphinothricin acetyltransferase (PAT), has also been tested as a plastid marker. When expressed in the nucleus, PAT confers resistance to the herbicide phosphinothricin (PPT) and is an excellent marker for the selection of nuclear gene transformants. Expression of the *bar* gene in plastids confers PPT resistance when

Figure 1

A transformed plastid genome is formed by two recombination events that are targeted by homologous sequences. The plastid genome segments that are included in the vector are marked as the left (LTR) and right targeting regions (RTR).



introduced by selection for a linked *aadA* (spectinomycin resistance) gene. However, *bar* was not found to be suitable for the direct selection of transplastomic lines, even when expressed at a high level (when PAT may accumulate to 7% of total soluble cellular protein [TSP]). Thus, it appears that the subcellular localization of the gene encoding the detoxifying enzyme PAT is crucial when directly selecting by herbicide resistance [14**].

A negative-selection scheme has been developed that is based on the expression of the bacterial enzyme cytosine deaminase in plastids [15]. Cytosine deaminase catalyzes the deamination of cytosine to uracil. 5-fluorocytosine is toxic to cells that express cytosine deaminase because this enzyme converts 5-fluorocytosine to the toxic 5-fluorouracil.

This negative selection scheme was utilized to identify seedlings on 5-fluorocytosine-medium from which *codA* was removed by the P1 bacteriophage site-specific recombinase CRE-lox [16**].

β -glucuronidase (GUS) and, more recently, green fluorescent protein (GFP) have been used as plastid reporter enzymes. The enzymatic activity of GUS can be visualized by histochemical staining [17], whereas GFP is a visual marker that allows the direct imaging of the fluorescent gene product in living cells. The GFP chromophore forms autocatalytically in the presence of oxygen and fluoresces green when absorbing blue or ultra-violet light. GFP has been used to detect transient gene expression [18] and stable transformation events [19,20] in chloroplasts. GFP

Figure 2

Plastid transformation vectors. (a) The pPRV vector family target insertions in the *trnV-rps12/7* intergenic region [4]. There is no read-through transcription of transgenes from the *rrn* operon. In the pPRV111 and pPRV112 series, *aadA* is expressed from alternative signals and the pUC vector MCS is available in both orientations. (b) Universal plastid vector [9**,10,11] targets insertions in the *trnI* and *trnA* intergenic region in the *rrn* operon. The insertion targets unique *XbaI* (X) and *SpeI* (Sp) sites in *TpsbA* derived from plasmid pZS197 [6]. The *aadA* gene is transcribed from two promoters: one directly upstream of *aadA* and a second upstream of the *rrn* operon. (c) The pRB94/pRB95 vectors target the *trnI-trnG* intergenic region. Vectors differ with respect to the orientation of the Bluescript plasmid MCS. No information has yet been published on read-through transcription at the MCS. Red, horizontal wavy lines represent transcripts; their thickness indicates the relative abundance of their mRNA. The plastid genes marked are: *ORF70B*, *psaB*, *psbD*, *rrn16*, *trnV*, *rps12/7*, *rps14*, *trnA*, *trnM*, *trnG*, *trnI*, *trnS* and *ycf9*. Restriction enzyme recognition sites are labeled *BglI* (B), *EcoRI* (RI), *HindIII* (H), *PstI* (P), *SpeI* (Sp), *StuI* (S) and *XbaI* (X). Restriction sites removed during construction are in brackets. PL cassettes are symbolized with boxes shaded with horizontal lines. T cassettes are shaded with vertical lines.

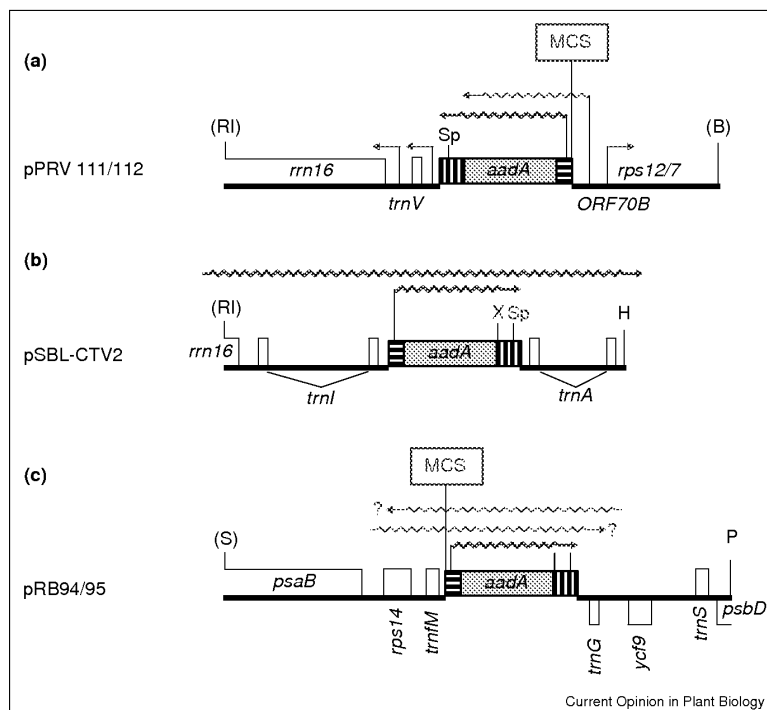
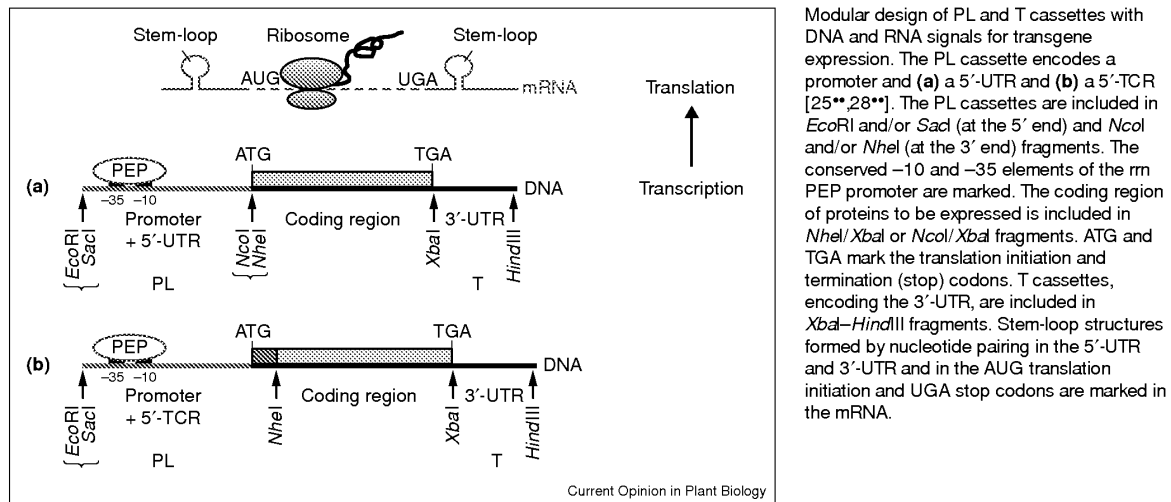


Figure 3



has also been fused with AAD and used as a bifunctional visual and selective marker gene [21].

Expression cassettes

To obtain high levels of protein expression several criteria must be met. First, the mRNA must accumulate to a high level as ensured by a strong promoter and stable mRNA. mRNA stability is determined by the 5' untranslated mRNA region (5'-UTR) and the 3'-UTR. Second, the mRNA must be translated efficiently. The rate-limiting step of translation is its initiation, which is facilitated by the 5'-UTR. The 5'-UTR is involved in mRNA-rRNA interactions (between the mRNA ribosome-binding site and 16S rRNA 3'-end) and interactions with translational-activating proteins that facilitate loading onto ribosomes. Third, the protein should be stable.

Plastid expression cassettes consist of a 5'-regulatory region (PL cassette) and a 3'-regulatory region (T cassette) and have convenient restriction sites at their boundaries to facilitate cloning (Figure 3). The PL cassette includes a promoter and a transcribed region to control translation. The translation control sequences may be the mRNA 5'-UTR (Figure 3a) or the 5' translation control region (5'-TCR), which includes the 5'-UTR and the amino-terminus of the coding region (Figure 3b). Promoters in the plastid genome are recognized by the multisubunit plastid-encoded plastid RNA polymerase (PEP) or the phage-type nucleus-encoded plastid RNA polymerases (NEP) [22•]. Most studies have focused on the strong sigma⁷⁰-type PEP promoter of the *rrn* operon promoter (Prn). Prn can be fused with translation control sequences of plastid and phage origin to facilitate the translation of recombinant proteins. The 5'-UTR of Prn derivatives listed in Table 1 are truncated and mutant forms of native plastid or phage translation control sequences.

The T cassette encodes the mRNA 3'-UTR, typically including a stem-loop structure (Figure 3). The 3'-regulatory region is important for mRNA stability and functions as an inefficient terminator of transcription [23]. Most commonly used T cassettes derive from the plastid *psbA*, *rbcl* and *rps16* genes (Table 1). The *psbA* and *rbcl* 3'-UTRs appear to yield more stable mRNAs than the *rps16* 3'-UTR.

Manipulation of protein levels by engineering the mRNA 5'-UTR and coding region amino-terminus

Expression levels from Prn derivatives vary from low protein levels (0.001% of TSP) up to 45% of TSP, underlining the importance of posttranscriptional regulation in plastid gene expression. Protein yield depends on the mRNA sequence, which gives rise to secondary structures and interacts with the 16S rRNA 3'-end and translation-activating proteins [24]. Specific PLrn cassettes tend to yield consistently high (or low) protein levels when expressing different proteins (Table 1). The inclusion of the amino-terminus of the coding region of the source plastid gene tends to enhance protein accumulation (by approximately two-fold), probably because the sequences around the AUG translation initiation codon have evolved together to ensure the translation of the encoded protein. For example, neomycin phosphotransferase II (NPTII) comprised 10.8% and 4.7% of TSP when expressed from a cassette including the *rbcl* (pHK34/pHK35) construct pair with and without a coding region amino-terminal segment, respectively. For the *atpB* (pHK30/pHK31) construct pair, these values were 7.0% and 2.5%, respectively. Silent mutations in the amino-terminus of the coding region reduced translation efficiency causing a 35-fold reduction in the NPTII levels (from 10.8% of TSP to 0.31%) [25•]. The opportunity to improve protein expression by manipulating the amino-terminus of the coding-region has been

Table 1.

Examples of high-level accumulation of recombinant proteins in chloroplasts expressed from plastid transgenes with Prm promoter derivatives*.

Protein	TSP concentration	Plasmid	Promoter	5'-UTR	Amino-terminal fusion	Coding region	3'-UTR	Reference
NPTII	1.0%	pTNH32	Prm	<i>rbcl</i>	<i>rbcl</i> ; 5 AA	<i>neo</i>	<i>psbA</i>	[12]
NPTII	2.5%	pHK31	Prm	<i>atpB</i>	—	<i>neo</i>	<i>rbcl</i>	[48]
NPTII	7.0%	pNK30	Prm(1)	<i>atpB</i>	<i>atpB</i> ; 14 AA	<i>neo</i>	<i>rbcl</i>	[25**]
NPTII	4.7%	pHK35	Prm	<i>rbcl</i>	—	<i>neo</i>	<i>rbcl</i>	[48]
NPTII	10.8%	pHK34	Prm(2)	<i>rbcl</i>	<i>rbcl</i> ; 14 AA	<i>neo</i>	<i>rbcl</i>	[25**]
NPTII	23.0%	pHK40	Prm	<i>T7g10</i>	—	<i>neo</i>	<i>rbcl</i>	[28**]
NPTII	16.5%	pHK38	Prm	<i>T7g10</i>	<i>T7g10</i> ; 14 AA	<i>neo</i>	<i>rbcl</i>	[28**]
EPSPS	0.3%	pMON38798	Prm	<i>T7g10</i>	—	CP4	<i>rps16</i>	[26**]
EPSPS	>10.0%	pMON45259	Prm	<i>T7g10</i>	GFP; 14 AA	CP4	<i>rps16</i>	[26**]
GFP	5.0%	pMON30125	Prm(3)	<i>rbcl</i>	—	<i>gfp</i>	<i>rps16</i>	[19]
GFP	5.0%	MR220	Prm(3)	<i>rbcl</i>	—	<i>gfp</i>	<i>rps16</i>	[20]
AAD-GFP	8.0%	pMSK56	Prm(1)	<i>atpB</i>	<i>atpB</i> ; 14 AA	<i>AadA-gfp</i>	<i>psbA</i>	[21]
AAD-GFP	18.0%	pMSK57	Prm(2)	<i>rbcl</i>	<i>rbcl</i> ; 14 AA	<i>AadA-gfp</i>	<i>psbA</i>	[21]
Ubiquitin-somatotropin	7.0%	pMON38794	Prm	<i>T7g10</i>	—	—	<i>rps16</i>	[27**]
PAT	>7.0%	pKQ3	Prm(1)	<i>atpB</i>	<i>atpB</i> ; 14 AA	<i>s-bar</i>	<i>rbcl</i>	[14**]
PAT	>7.0%	pKQ18	Prm(1)	<i>atpB</i>	<i>atpB</i> ; 14 AA	<i>b-bar2</i>	<i>rbcl</i>	[14**]
Cry1Ac	5.0%	pZS224	Prm(3)	<i>rbcl</i>	—	<i>cry1A(c)</i>	<i>rps16</i>	[38]
Cry2Aa2	3.0%	pZS-KM-cry2A	Bt	—	—	<i>cry2Aa2</i>	?	[8]
Cry2Aa2, 20 kDa; 29 kDa; CTB	45.3%	pLD-BD Cry2Aa2	2x-Prm	Bt	—	<i>orf11-orf2-ery2Aa2</i>	<i>psbA</i>	[9**]
CTB	4.1%	pLD-LH-CTB	2x-Prm	Synthetic GGAGG	—	<i>ctxB</i>	<i>psbA</i>	[11]

*Identical Prm derivatives (PL cassettes) in different constructs are identified by arbitrary number. Prm derivatives that are not numbered are unique in some detail of the PL cassette. Same origin of 5'-UTR does not mean identical 5'-UTR sequences. For example, plasmids pTNH32 and pHK35 are listed to encode a *neo* gene with a *rbcl* 5'-UTR. However, the actual *neo* mRNA 5'-UTR encoded in plasmid pTNH32 includes 18 nucleotides of *rbcl* 5' UTR fused with 34 nucleotides of the *rrn* transcript, whereas the pHK35-encoded *neo* 5'-UTR includes 58 nucleotides of the 182-nt native *rbcl* 5'-UTR. AA, amino acids.

demonstrated by a more than 30-fold increase in CP4 EPSPS accumulation after fusion with 14 amino-terminal amino acids of GFP [26**]. Fusion of the human somatotropin with ubiquitin [27**] and GFP with AAD [21] increased levels of protein accumulation. This may be due to enhanced translation rates, enhanced protein stability or both.

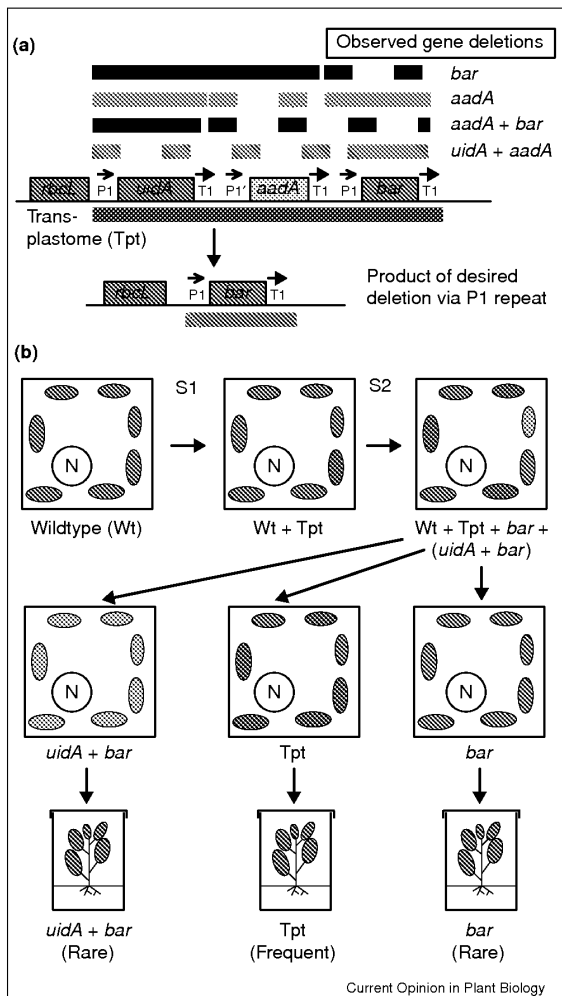
Protein accumulation from heterologous translation control signals incorporated with the heterologous coding region depends on their recognition in chloroplasts. Translation signals controlling the *Bacillus thuringiensis cry2Aa2* operon were efficiently recognized, yielding 45.3% TSP as Cry2Aa2 protein crystals [9**]. Translation control signals may only be part of the reason for this high-level expression as the co-expression of two helper proteins alongside the protoxin facilitated the formation of stable protein crystals. Levels of expression of the Cry2Aa2 protein alone (without the helper protein, 2–3% of TSP) [8] and cholera toxin B subunit (4.1% of TSP) [11] were satisfactory, but not unusually high. The low level of a protein-based polymer from a different RBS-coding region segment, despite the high level of mRNA encoding it, is a reminder that it may be risky to include untested translation control elements from a heterologous source [10].

Although the plastid expression system is, in general, prokaryotic, the rules of prokaryotic gene expression do not always apply to it. Increasing complementarity between sequences downstream of the T7-phage gene 10 (T7g10) AUG and the penultimate stem of the 16S rRNA 3'-region reduced NPTII accumulation 100-fold by reducing translation efficiency and destabilizing the NPTII mRNA [28**]. In *E. coli*, similar changes would enhance rather than reduce protein accumulation.

Expression of heterologous proteins: codon usage and mRNA stability

The expression of proteins encoded by eukaryotic nuclear genes in *E. coli* is often problematic because of differences in codon usage and/or degradation of the eukaryotic mRNA. In tobacco plastids, with the exception of the borderline CGC codon (which appears 3.9 times per 1000 codons), all codons are used more frequently than 4.3 times per 1000 codons, a frequency below which problems were encountered when expressing heterologous proteins in *E. coli*. Indeed, codon optimization in plastids has so far yielded at best a few-fold increase in protein yield. Protein accumulation from a synthetic, codon-optimized version of CP4 (containing 77% plastid-preferred

Figure 4



Elimination of marker genes by loop-out via short, directly repeated sequences. (a) Map of the transformed plastid genome with a reporter gene (*uidA*), a selective marker (*aadA*) and a herbicide resistance gene (*bar*). P1 promoter fragments (of 174 basepairs) and the 418-basepair *TpsbA* cassettes (T1) are short, direct repeats. Breaks in the black, orange and blue bars represent gene deletions detected by Iamtham and Day [29]. (b) Multiple, alternative recombination events and segregation of the plastid genome yield marker-free herbicide resistant plants. Note that selection for spectinomycin resistance (S1) may be followed by selection for herbicide resistance (S2) that eliminates the non-herbicide resistant deletion derivatives. N, nucleus.

codons) was just 1.5–2.0-fold higher than from the bacterial CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) coding region (which contains 44% plastid-preferred codons) [26••].

Neither codon usage nor mRNA stability was reported to be a problem when expressing the human somatotropin cDNA in chloroplasts. Protein levels were reasonably high

(7% of TSP) as long as the somatotropin-coding region was translationally fused with ubiquitin and expressed from a modified T7g10 leader [27••]. Data on further human proteins are required to fully assess the problems associated with expressing human cDNAs in chloroplasts.

Marker elimination systems

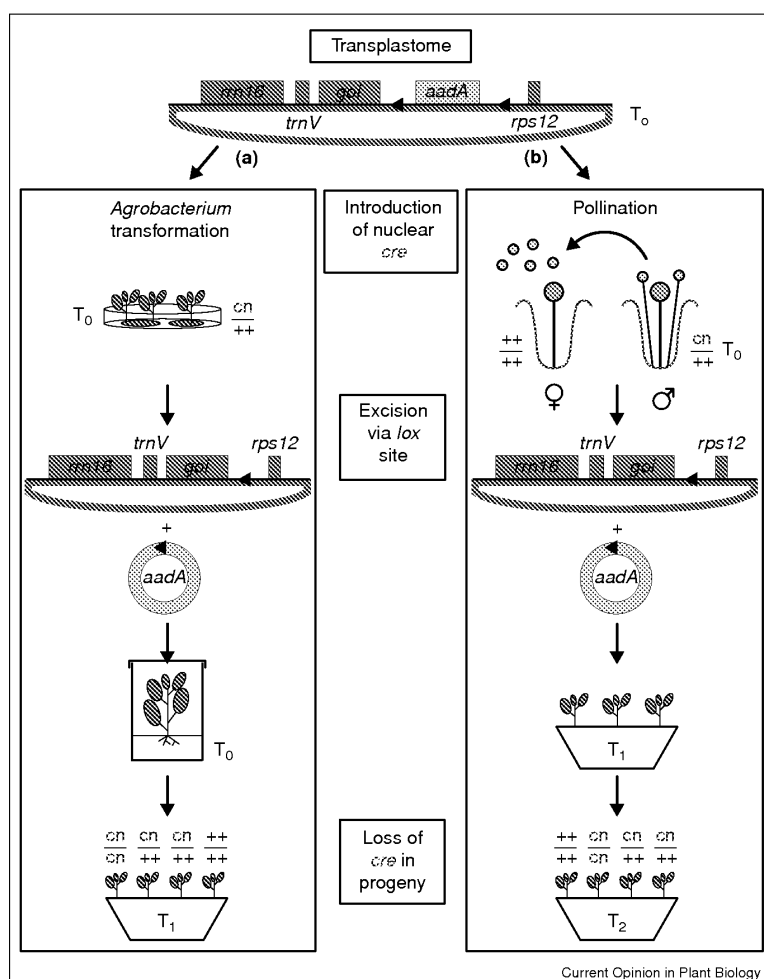
Expression of the selective marker at a high level may be desired to ensure selective amplification when only a few transformed genome copies are present. When the marker gene is present in each plastid genome, however, the marker protein may make up as much as 10% of TSP, a significant metabolic burden on the plant. In addition, given the concern about gene flow and the possible health hazards of antibiotic resistance genes, it is desirable to remove the selective marker once it has fulfilled its useful purpose.

The scheme for the removal of plastid markers developed by Iamtham and Day [29] exploits the formation of multiple recombination products in the same plastid (Figure 4). Plastids in this scheme are transformed with a construct in which the selective marker is flanked by short direct repeats. The repeats are two 174-basepair PLrrn promoter fragments (P1, light arrow) and three 418-basepair *TpsbA* sequences (T1, heavy arrow; Figure 4). P1' and P1 are also partially homologous. Heteroplasmic clones obtained after the delivery of DNA, in which different fortuitous deletion derivatives form by homologous recombination via the short direct repeats, are identified by spectinomycin resistance. Derivatives that lack the marker gene but carry the gene of interest are identified by herbicide resistance and DNA-gel-blot analyses after plant regeneration. The difficulty of this approach is that transformation and the elimination of marker genes occur simultaneously, making the process laborious and difficult to control.

Transformation and marker gene elimination are separate processes in the CRE-*lox* plastid marker elimination system developed independently by Hajdukiewicz *et al.* [30••] and in our laboratory [16••] (Figure 5). CRE is a phage site-specific recombinase that efficiently excises any sequence between two directly oriented 34-basepair *lox*-sites. According to the CRE-*lox* scheme, the marker gene (flanked by two directly-oriented *lox* sites) and the gene of interest are introduced into the plastid genome in the absence of CRE activity. The transformed plastid genome is stable until CRE activity is provided from a nuclear-encoded, plastid-targeted CRE. The nuclear *Cre* may be introduced by *Agrobacterium*-mediated transformation [16••,30••] or by pollination [16••], and is subsequently removed by segregation in the seed progeny. Although the desired marker-free plants can be readily obtained by this system, unpredicted rearrangements of the plastid genome were detected in a significant fraction of the *Agrobacterium*-transformed clones. It appears that these rearrangements are much less frequent in plants obtained by pollination.

Figure 5

Scheme for the elimination of marker genes using the CRE-lox site specific recombination system in plastids. In this example, the transplastome contains the marker gene *aadA* flanked by *lox* sites (arrowheads). The gene of interest (*goi*) that was introduced by selection for the marker gene is shown in blue. Introduction of *cre* (a) by *Agrobacterium* transformation or (b) by crossing results in expression of the CRE site-specific recombinase from the nuclear *cre* gene. The CRE is plastid targeted, and will simultaneously excise the marker gene from all the plastid genome copies. In the experiment described in [16**], the gene to keep (i.e. the *goi*) was *aadA* and the gene to remove was *codA*, a negative selectable marker. In [30**], the gene to be eliminated was *aadA* and the gene to keep was *gfp* (encoding GFP). Presence of *cre* and the linked kanamycin-resistance (*neo*) gene in the nuclear genome are indicated by c and n, respectively; their absence by a '+'. T_0 refers to transgenic plants regenerated from tissue culture; T_1 and T_2 are the first and second generation progeny of T_0 produced by self pollination.



Applications of plastid transformation

Plastid transformation has been utilized in basic science, biotechnology and agronomy [1*,2,3]. Recently, progress has been made in engineering Rubisco, the carbon-dioxide-fixing enzyme. The long-term objective of Rubisco engineering is to replace the existing Rubisco with a more efficient form. Earlier attempts to replace the tobacco Rubisco with homologs from sunflower and the cyanobacterium *Synechococcus* PCC 6301 did not result in functional hybrid Rubisco [31]. When relocated to the plastid genome, a tobacco *RbcS* gene was capable of directing the synthesis of small Rubisco subunits. However, these subunits were not abundant, suggesting that either the translation of the mRNA or the access of the product to the Rubisco assembly pathway was not efficient [32]. Rubisco translated from inserted *rbcLS* operons from two non-green algae were insoluble [33]. The first success in Rubisco engineering was the replacement of the tobacco *rbcL* gene,

which encodes the Rubisco large subunit, with the Form II Rubisco of *Rhodospirillum rubrum*, a photosynthetic bacterium. The transplastomic tobacco plants require carbon dioxide supplementation, consistent with the kinetic properties of the bacterial Rubisco [34**]. These transplastomic tobacco plants are the first photosynthetic higher plants with a foreign Rubisco that are fully autotrophic and fertile. They represent a milestone on the road towards achieving the long-term goal of engineering improved photosynthetic efficiency.

Metabolic engineering through transformation of the plastid genome has also been reported. To boost tryptophan production, a feedback-insensitive tobacco anthranilate synthase alpha-subunit was overexpressed from a tobacco cDNA in plastids. Free tryptophan was 10-fold more prevalent in the transplastomic leaves. Although the transgene was highly expressed, the overall increase in

enzymatic activity was only four-fold, suggesting that the overexpression of both anthranilate synthase subunits is necessary to fully exploit the benefits of boosting this pathway [35]. The desire to increase the lipid content of seeds is the reason for attempts to boost acetyl-CoA carboxylase activity by introducing a stronger promoter upstream of *accD* by plastid transformation [36].

There is interest in using transgenic plants as a source of recombinant protein therapeutics and vaccines [37]. Production of human secretory protein somatotropin in tobacco chloroplasts was the first such application of plastid transformation. Proper protein folding and the formation of disulfide bonds normally require passage through the endoplasmic reticulum. However, the chloroplast-produced somatotropin (which comprised 7% of TSP) was in a biologically active, soluble and disulfide-bonded form, which was nearly devoid of complex post-transcriptional modifications [27•]. The mature form of the B subunit of the cholera toxin, a candidate vaccine antigen, has also been expressed in chloroplasts. The chloroplast-produced B subunit (which comprised 4.1% of TSP) assembled into oligomers and was biologically active in a cell-culture assay [11].

The most common transgenic traits in field grown plants are resistance to insects and herbicides. Both of these traits are expressed from nuclear genes but plastid versions of the insecticidal protein genes are also available [8,9•,38]. Although a petunia EPSPS gene has been expressed in plastids, the resulting level of glyphosate tolerance was below that required for field applications [7]. Recently, strong glyphosate resistance was engineered by the overexpression in plastids of tolerant forms of different prokaryotic (*Achromobacter*, *Agrobacterium* and *Bacillus*) EPSPS genes. Greater accumulation of plastid-expressed EPSPS protein than of EPSPS from nuclear genes was necessary to confer strong resistance [26•]. However, field-level tolerance to 'Liberty', a herbicide containing PPT as an active ingredient, was obtained by expression of *bar* in the plastid genome. In this case, resistance did not require high-level accumulation of PAT [14•]. Utilization of the plastid herbicide-resistance genes will have to wait until plastid transformation is achieved in major crops.

Implementation of plastid engineering technology in new crops

Plastid transformation in tobacco [6,39] was followed by plastid transformation in two other solanaceous species, potato [19] and tomato [5•]. Plastid transformation in tomato is a significant breakthrough as tomato fruits were shown to express relatively high levels (approximately 0.5% of TSP) of the marker gene product. Significant improvement in the expression level of recombinant proteins in tomato fruit is anticipated thanks to the use of new, more sophisticated expression tools. This is the first example of protein expression in an edible plant part, providing promise of high-level expression of oral vaccines [5•].

Plastid transformation in *Arabidopsis*, although feasible, is very inefficient [40], probably due to the inefficient incorporation of the transforming DNA. In rice, the plastid genomes of embryogenic cells could be transformed relatively easily. However, plant regeneration from cultured rice cells by standard protocols occurs prior to achieving the homoplasmic state, making it difficult to obtain genetically stable transplastomic plants [21]. Problems such as these that are specific to taxonomic groups, once recognized, can be overcome. Plastid transformation in *Arabidopsis* and rice will be followed by implementation of protocols in the related crops: oilseed rape, vegetable brassicas, maize, barley and wheat.

Conclusions and future directions

Tools, including vectors, expression cassettes and systems for the elimination of marker genes, are available for the expression of recombinant proteins in tobacco chloroplasts. These will be combined in a new generation of vectors to provide high-level protein expression and the convenient elimination of marker genes. The focus of research in tobacco will shift to issues related to protein modification, processing and subunit assembly. Tools for regulated plastid gene expression are yet to be developed. A T7-phage RNA polymerase expressed in the nucleus from a chemically inducible promoter, and driving the expression of transgenes in plastids from a T7 gene 10 promoter, is the first system to address this problem [2,41].

Advantages of incorporating the transgenes in the plastid genome are: containment of transgenes due to the lack of pollen transmission; expression of multiple genes in operons; high expression levels; possibility of expressing unmodified bacterial genes and human cDNAs; and lack of gene silencing and position effects. The disadvantages are that the proteins are not exported and the difficulty of the technology required. To fully benefit from the technology, experience gained from work on tobacco should be applied to all crops. The discovery of the right combination of tissue culture procedures and selective markers will be crucial for the success of plastid engineering. Will it be necessary to construct vectors for each crop? Probably not, although specific vectors may be constructed for each of the larger taxonomic groups. Plastid transformation in potato [19] and tomato [5•] using tobacco vectors is encouraging in this regard. There is no need for perfect homology between the recipient genome and transformation vector as multiple internal exchange events result in the mosaic-like integration of donor DNA [42]. The advantage of using homologous targeting sequences is that only one type of transplastome may form. Species-specific differences in recognition of DNA and RNA signals and in capacity to alter essential mRNA editing sites will determine how widely the vectors can be used.

Nuclear transgene flow via pollen to wild weedy relatives can be a problem, especially if gene transfer enhances the fitness of the weedy relative by conferring resistance to

herbicides and insect pests [43]. The incorporation of transgenes into the plastid genome would provide significant advantages, as plastids in the major crops are not transmitted by pollen. Although the transgenes would not leave the transgenic crop in pollen, rare hybrids still could form if the crop were to be pollinated by the wild relatives or in mixed stands [44]. Relevant in this regard is that, of the 13 most important agricultural crops, 12 have a sexually compatible weedy relative with which they form a hybrid in some geographic area. An alternative source of plastid genome transfer to weeds could occur occasionally: paternal transmission of plastids has been detected under extreme selection pressure in a tissue-culture system in plants with an alien cytoplasm or in inter-specific crosses, even in species with a strict maternal inheritance of plastids [45,46]. Knowing the effort required to achieve the homoplasmic state in plastid transformation experiments [47], even if transfer occurs, such plastids are unlikely to become established in the recipient weed. Nevertheless, the possibility of genome transfer from the new transplastomic crops to weedy relatives will have to be evaluated.

Acknowledgements

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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)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R/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 Tn501-like transposon. Furthermore, two copies of a sequence similar to IS91-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 IS91-like elements. Two other dichlorprop-degrading bacterial strains, *Rhodospirillum rubrum* 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 (α KG)-dependent dioxygenase, which is encoded by *tfdA* (13, 20, 39, 45, 59). The presence of the functionally essential motif HX(D/

E)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R/K indicates that TfdA is related to several other (referred to as group II) α KG-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 α KG-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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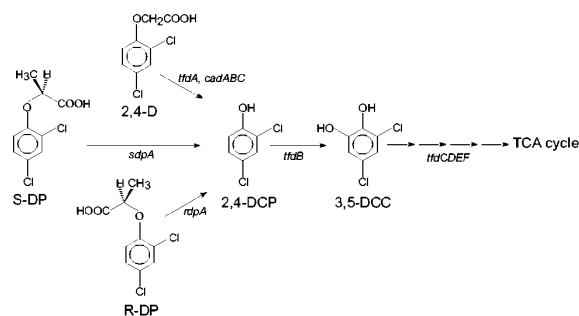


FIG. 1. Modified *ortho*-pathway for the degradation of chlorinated phenoxyalkanoate herbicides. 2,4-D, 2,4-dichlorophenoxyacetate; R-DP and S-DP, *R*- and *S*-enantiomers of 2(2,4-dichlorophenoxy)propionate, respectively; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; TCA, tricarboxylate.

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 *Rhodospirillum rubrum* 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- μ m-pore-size Minisart cellulose acetate filter (Sartorius) and added at a concentration of 100 μ g/ml.

Mercury resistance was examined on LB agar plates containing 0.1 mM HgCl₂, 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 miniTn5 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 (OD₆₀₀) of 1.0. Equal volumes of the donor and the recipient were mixed, and 100 μ l 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 μ l of the 10⁻³ to the 10⁻⁵ dilutions was plated on selective MM agar plates containing 100 μ g 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 TfdB, 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 μ l 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 *Rhodospirillum rubrum* strain P230 were amplified with primers rdp.fg (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 Tn5 transposon insertion sites were amplified by ST-PCR (6). To each sample either Tn5.outF (5'-GGC AGA TCT GAT CAA GAG-3' [27]) annealing to the I end of the transposon or Tn5.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); GGC CT-3'; FP-2, 5'-AGT TCA AGC TTG TCC AGG AAT TC(N); GCG CT-3'; FP-3, 5'-AGT TCA AGC TTG TCC AGG AAT TC(N); CCG GT-3'; and FP-4, 5'-AGT TCA AGC TTG TCC AGG AAT TC(N); 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 IS91-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

TABLE 1. Degenerate primers designed for the amplification of *rdpA* and *sdpA*

Peptide or primer	Sequence ^a	Position ^b
RDP_internal sequence 2 rdp_r	W E T L S P T M Q A T I E G L N 3'-AC GTY CGN TGN TAI CTY CC-5'	162-177
RDP_internal sequence 3 rdp_fA ^c rdp_fG ^c	N E I L D A F H T Y Q V I Y F P 5'-GCI TTY CAY ACN TAY CAA GT-3' 5'-GCI TTY CAY ACN TAY CAG GT-3'	43-58
SDP_N terminus sdp_fC ^c sdp_fI ^c	M Q T T L Q I T P T G A T L G A 5'-ATG CAR ACI ACI CTG CAR ATC AC-3' 5'-ATG CAR ACI ACI CTG CAR ATT AC-3'	1-16
SDP_internal sequence 1 sdp_rA ^c sdp_rG ^c	L G H V Q Q A G S A Y I 3'-GTA CAN GTY GTY CGN CC-5' 3'-GTA CAN GTY GTY CGN CC-5'	193-204

^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).

tfpD gene fragments was performed as described in reference 35. Fragments of *tfpD* and *tfpF* 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 H₂O, 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* XLI-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 (*tfpD*, *tfpD*, *tfpE*, and *tfpF* probes), 55°C (*rdpA* probe), 60°C (probes against *tfpD*, the *mer* operon, and the IS91 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-

TABLE 2. Sequence identities of the dichlorprop-cleaving enzymes RdpA and SdpA to other group II α KG-dependent dioxygenases

α -KG dioxygenase ^a	Strain, accession no.	% Identities ^b to	
		RdpA	SdpA
TfdA	<i>Ralstonia eutropha</i> , A27082	32/279	37/244
TauD	<i>Pseudomonas aeruginosa</i> , NP_252624	35/279	27/258
AtsK	<i>Pseudomonas putida</i> , AAD31784	31/296	31/296
YSD	<i>Saccharomyces cerevisiae</i> , Q12358	29/279	27/304
PvcB	<i>Pseudomonas aeruginosa</i> , AAC21672	20/298	21/277
SyrI	<i>Pseudomonas syringae</i> , AAB63253	17/323	18/308
CarC	<i>Pectobacterium carotovorum</i> , AAD38231	16/285	17/275
γ -BBH	<i>Pseudomonas</i> sp. strain AK-1, P80193	16/309	17/291
CS2	<i>Streptomyces clavuligerus</i> , AAA26723	16/208	17/275

^a TfdA, 2,4-D/ α KG dioxygenase; TauD, taurine/ α KG dioxygenase; AtsK, putative α KG-dependent alkylsulfatase; YSD, sulfonate/ α KG dioxygenase; PvcB, pyoverdine biosynthesis enzyme; SyrI, syringomycin biosynthesis enzyme I; CarC, carbapenem biosynthesis enzyme C; γ -BBH, γ -butyrobetaine hydroxylase; CS2, clavaminic synthase 2.

^b That is, the percent sequence identity over the total number of amino acids examined.

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 α KG-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-Tn5 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 Tn501 subgroup of the Tn3 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

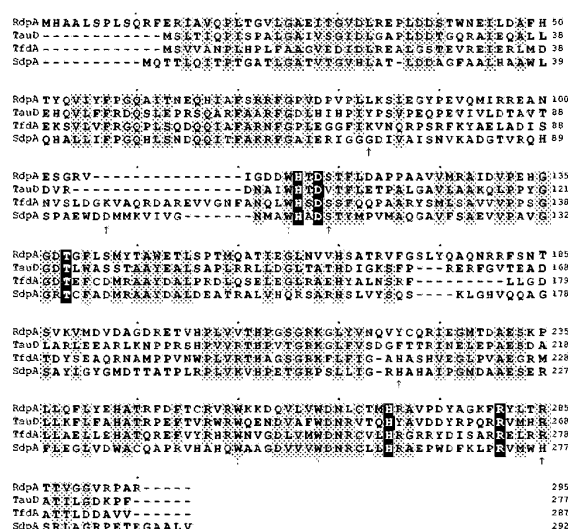


FIG. 2. Sequence alignment of RdpA and SdpA with other group II α KG-dependent dioxygenases. TfdA, *R. eutropha* 2,4-D/ α KG dioxygenase (accession no. A27082); TauD, *P. aeruginosa* taurine/ α KG dioxygenase (accession no. NP_252624). Conserved Fe(II)- and α KG-binding residues are shaded in black; 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.

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 Tn501 transposon showed 81% nucleotide sequence identity to a putative Tn3 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.

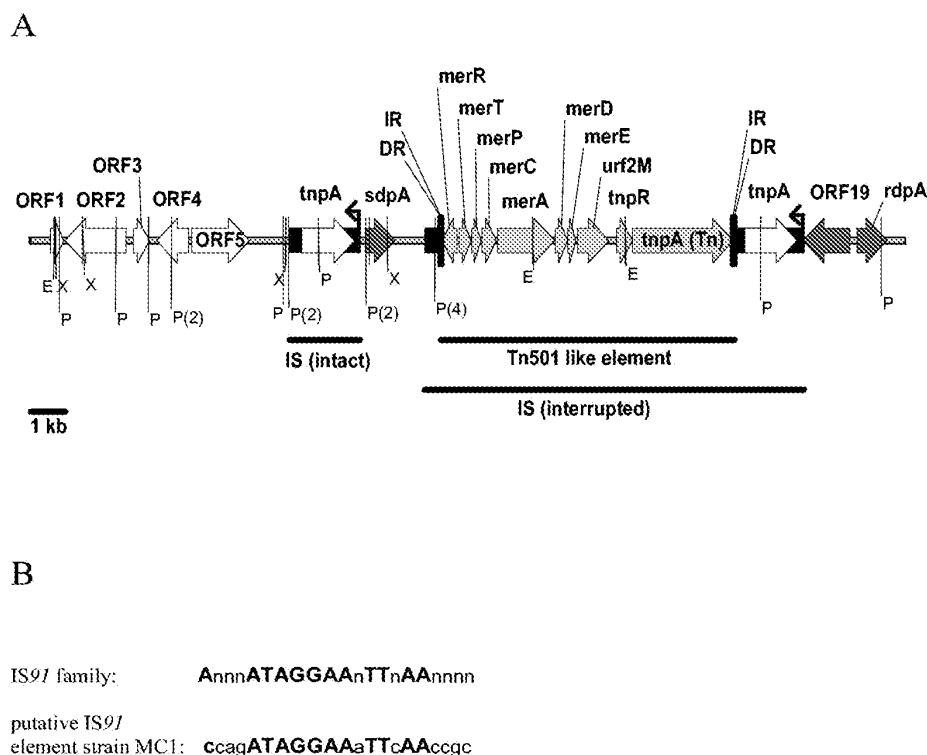


FIG. 3. Organization of *rdpA* and *sdpA* within the genome of strain MC1. (A) Genetic map of the genomic fragment containing *rdpA* and *sdpA*. For ORFs showing a high degree of similarity to other genes (>80% identity of the deduced amino acid sequence) the respective names are given. The gene designations 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 Tn501-like transposon, DR, 5-bp direct repeat. Two putative IS91-like elements are shown as black boxes; the positions of the *oriIS* are indicated as arrows above the sequence. The right IS91-like element is interrupted by a Tn501-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 IS91 family *oriIS* consensus sequence. Conserved nucleotides are shown in bold uppercase letters.

Furthermore, two copies of a putative IS element resembling the IS91/IS801 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 *oriIS*, the origin of replication, and a *terIS*, 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 Tn501-like element (Fig. 3A). The elements were 2,037 bp (intact copy) and 2,084 bp (interrupted copy) and contained an IS801-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 *oriIS* of this group of insertion elements (49) in all but the first nucleotide (Fig. 3B). Neither the termination sequence *terIS* nor the tetramers GTTC/CTTG for which IS91 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

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	<i>Shewanella oneidensis</i> MIR-1	65 (81/124)	130/128	AA553170.1
2	1006-2799	c	Glutathione-regulated potassium-proton antiporter	<i>Pseudomonas putida</i> KT2440	69 (385/556)	597/581	AEO16523.1
3	3018-3494	d	Putative bacterioferritin	<i>Delftia acidovorans</i>	70 (112/158)	158/158	CAC83620.1
4	3701-4642	c	Hypothetical protein, similar to LysR-type regulators	<i>Ralstonia metallidurans</i>	51 (161/314)	313/360	ZP_00022545.1
5	4745-6438	d	Putative dihydroxy acid dehydratase	<i>Ralstonia solanacearum</i>	76 (420/555)	564/557	CAD16119.1
6	7981-9528	d	Putative transposase, similar to IS801 transposase	<i>Pseudomonas</i> sp. strain ADP	99 (512/515)	515/515	AAK50272.1
7	9836-10714	d	2,4-D dioxygenase (TfdA)	<i>Ralstonia eutropha</i> JMP134	37 (92/249)	292/287	AZ7082
8	12107-12541	c	Mercury resistance regulatory protein (MerR)	<i>Enterobacter aerogenes</i>	97 (140/144)	144/144	CAA83896.1
9	12613-12963	d	Mercury uptake protein (MerF)	<i>Alcaligenes</i> sp.	96 (112/116)	116/116	CAA70196.1
10	12977-13251	d	Mercury uptake protein (MerP)	<i>Nitrosomonas europaea</i> ATCC 19718	94 (86/91)	91/91	CAA70197.1
11	13279-13701	d	Mercury uptake protein (MerC)	<i>Nitrosomonas europaea</i> ATCC 19718	86 (121/140)	140/140	CAD84751.1
12	13741-15426	d	Mercury ion reductase (MerA)	<i>Klebsiella pneumoniae</i>	88 (494/561)	561/561	AAM44226.1
13	15444-15809	d	Mercury resistance secondary regulatory protein (MerD)	<i>Pseudomonas</i> sp. strain ADP	95 (115/121)	121/121	AAK50284.1
14	15806-16042	d	Putative protein (MerE)	<i>Pseudomonas</i> sp. strain ADP	91 (71/78)	78/78	AAK50283.1
15	16093-17028	d	Putative protein, similar to Urf2M	<i>Ralstonia metallidurans</i>	88 (274/310)	311/329	CAD97548.1
16	17245-17718	d	Resolvase (TnpR)	<i>Nitrosomonas europaea</i> ATCC 19718	98 (154/157)	158/186	CAD84747.1
17	17721-20690	d	Tn3 family transposase (TnpA)	<i>Nitrosomonas europaea</i> ATCC 19718	95 (945/989)	989/989	CAD84746.1
18	21024-22571	d	Putative transposase, similar to IS801 transposase	<i>Pseudomonas</i> sp. strain ADP	99 (511/515)	515/515	AAK50727.1
19	22752-24137	c	Putative aromatic compound MFS transporter	<i>Pseudomonas putida</i> KT2440	73 (318/433)	461/450	AA69258.1
20	24330-25217	d	Taurine dioxygenase (TauD)	<i>Pseudomonas aeruginosa</i> 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 IS91-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 *Rhodospirillum rubrum* 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 α KG-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 α KG-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)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R/K, which is conserved throughout group II α KG-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 α KG-binding residues Thr141 and Arg274 (Fig. 2). All of these residues were conserved in RdpA and SdpA, representing the putative Fe(II) and α KG-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

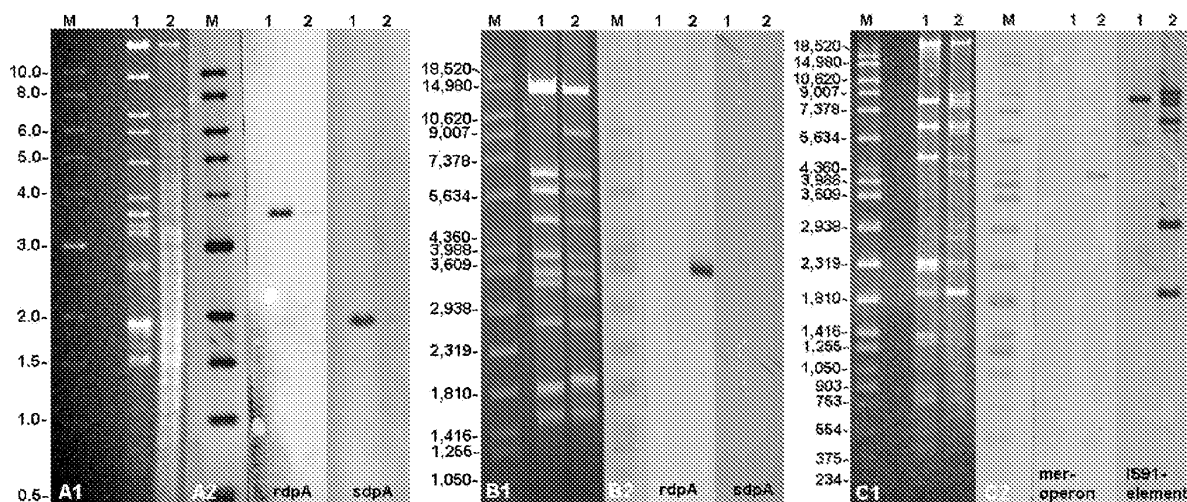


FIG. 4. Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of plasmid pMC1 with pMC1-DP⁻, the plasmid of mutant MC1-DP⁻. (A) Localization of *rdpA* and *sdpA* within the genome of strain MC1. Plasmid DNA (lane 1) and genomic DNA (lane 2) was digested with PstI, 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 IS91 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 α KG dioxygenases a high degree of structural conservation was observed between clavaminic 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/TfdA α 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 α KG dioxygenase family.

Transposon mutagenesis inactivation of *rdpA* indicated that it was a single-copy gene and gave a further proof of its func-

tionality 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 *Rhodospirillum rubrum* 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

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 IS91-like element was found to be considerably reduced. This suggests that recombination between IS91 elements could lead to the deletion of one or both dioxygenase genes and the Tn501-like transposon. Although IS91-like elements do not cause deletion of regions adjacent to them, a deletion of a genomic fragment flanked by IS91-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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ERRATUM

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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L3	233	aryloxyalkanoate adj dioxygenase	USPAT	OR	OFF	2016/12/29 10:26
L4	0	l3 and @PD< "20051028"	USPAT	OR	OFF	2016/12/29 10:27
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Isolation of rapeseed genes expressed early and specifically during development of the male gametophyte

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Abstract

A cDNA subtraction and differential hybridization strategy was used to isolate cDNAs expressed early during male gametophyte development in the important crop species *Brassica napus*. Three cDNAs, corresponding to genes highly and specifically expressed at the tetrad and microspore stages, are presented here. The analysis of one of them, named *BnM3.4*, by *in situ* hybridization, showed that it is expressed specifically and at a high level in the rapeseed microspore. The specificity in its profile of expression is most likely transcriptionally controlled as a similar pattern of expression was also observed in *Arabidopsis thaliana* plants transformed by the *BnM3.4* promoter fused to the reporter GUS-coding sequence. The putative *BnM3.4* promoter contains three dispersed copies of a motif described previously in the promoters of several genes expressed in the male gametophyte. The *BnM3.4* gene encodes a predicted novel proline-rich protein of 23.4 kDa which may interact with cytoskeletal components or have a structural role in the cell wall.

Introduction

Pollen development in angiosperm plants occurs in a specialized floral organ, the anther. It starts by a series of mitotic divisions of archeosporial cells from the primary sporogenous layer leading to the formation of meiocytes. Each meiocyte undergoes meiosis to generate a tetrad of four haploid microspores surrounded by a callose wall, whose digestion then liberates them into the anther locule. A complex extracellular matrix composed of intine and exine is built around the male gametophyte after microspore differentiation. An asymmetric cell division of each microspore produces the bicellular pollen grain in which the large vegetative cell encloses the smaller generative cell. This latter cell has a condensed nucleus and a reduced cytoplasm. In the majority of plant species, the second mitotic division of the generative cell occurs after pollination

within the growing pollen tube producing two sperm cells. In some genera such as *Brassica* this second pollen mitosis occurs earlier during pollen maturation. The pollen grain is hence released from the anther locule in a tricellular form. This developmental programme is necessary to prepare the pollen grain for efficient interaction with the stigma, for rapid germination and pollen tube growth, and for successful delivery of sperm cells to the ovules (Bedinger *et al.*, 1994).

Despite the wealth of descriptive studies on the structural and physiological aspects of pollen formation, our understanding of the molecular events remains rather limited. Our main goal is to extend our knowledge on microspore differentiation and development through the identification and characterization of novel genes specifically expressed during early stages of microgametogenesis. Such genes may also provide alternative methods for controlling male fertility in economically important plants, such as in our case *Brassica napus*. To identify genes that might be

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF136223 (*BnM3.4*).

implicated in the developmental process, differential screening of cDNA libraries constructed from whole anthers has been the most commonly used approach. A number of genes have been shown to be expressed in developing pollen, as well as in the tapetum or other sporophytic tissues of the anther (Ursin *et al.*, 1989; Koltunow *et al.*, 1990; Nacken *et al.*, 1991; Theerakulpisut *et al.*, 1991; Paul *et al.*, 1992; Aguirre and Smith, 1993; Robert *et al.*, 1993; Bucciaglia and Smith, 1994; Ross and Murphy, 1996). Some pollen-specific genes expressed after the first mitotic division and presumably playing a role in pollen development, maturation, germination or pollen tube growth have also been described (Hanson *et al.*, 1989; Brown and Crouch, 1990; Shen and Hsu, 1992; Weterings *et al.*, 1992; Brander and Kuhlemeier, 1995; Stanchev *et al.*, 1996; Yu *et al.*, 1998). Finally, so far, a single tobacco gene has been identified that shows a microspore-specific expression (Oldenhof *et al.*, 1996).

We report the isolation of cDNAs from *B. napus* that are expressed during early pollen development. During this work, we developed a novel combined approach by differential screening of a microspore cDNA library with a subtracted probe enriched for microspore-specific sequences. We describe here in detail the characterization of the structure and expression of the *BnM3.4* gene corresponding to the microspore-specific cDNA M3.

Materials and methods

Plant material

Plants of *B. napus* L. cv. Brutor were grown in an open field or under standard greenhouse conditions and were used for RNA and DNA isolation. *Arabidopsis thaliana* (Wassilevskija ecotype) plants used for transformation (Bechtold *et al.*, 1993) were grown in a greenhouse under standard conditions. Transgenic *A. thaliana* T₁ seedlings were selected in a greenhouse on sand, sub-irrigated with water containing Basta herbicide (7.5 mg/l phosphinothricine). Two months later, T₂ seeds were harvested individually. *In vitro* culture of seedlings for segregation analysis was done in a culture chamber on *A. thaliana* medium (Estelle and Somerville, 1987) containing 5 mg/l of phosphinothricine as selective agent.

RNA isolation and poly(A)⁺ RNA purification

Several grams of rapeseed flower buds from 0.2 mm to more than 4 mm in length were harvested in 4 different classes according to bud length. Bud length was measured from the base to the tip of the outermost sepal. Male gametophytes from graded floral buds were isolated and purified as previously described (Albani *et al.*, 1990) with a few modifications. After disruption of the buds in a blender with a solution of 10% sucrose (pH 7), the resulting suspension was filtered through 80 µm nylon mesh. Only the suspension containing microspores (from 2 to 3 mm buds) was filtered through 45 µm nylon mesh. After two washes, as described by Albani *et al.* (1990), the pellet was frozen in liquid nitrogen and stored at -80 °C if not immediately used for RNA extraction. The purified male gametophytes from the different classes of bud were disrupted by 3 cycles of pressurization (at 110 bar) depressurization in a mini-bomb (Bioblock Scientific, Illkirch, France) in 50% RNA extraction buffer and 50% phenol before performing RNA extraction as previously reported (Dean *et al.*, 1985).

Poly(A)⁺ RNAs were purified from total RNA using the mRNA purification kit from Pharmacia Biotech.

Total RNA was extracted from young rapeseed seedlings, total fertile and male-sterile buds, pistils, sepals and petals, roots, leaves and stems as described above.

cDNA library construction and cDNA subtraction

The procedure used for the isolation of organ-specific cDNA was based on an unpublished protocol from S. Lok and D.C. Baulcombe (Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich, UK). It is the result of several reports on ligation-mediated PCR (Mueller and Wold, 1989), direct incorporation of biotin nucleotide during PCR (Lo *et al.*, 1988) and differential removal of biotinylated DNA by a streptavidin/phenol extraction procedure (Sive and St John, 1988; Wang and Brown, 1991) and was modified as follows.

The blunted 'tracer' cDNA (T) and the 'driver' cDNA (D) were synthesized from 3 µg of poly(A)⁺ RNA from microspores and male-sterile buds (Ogu-INRA) (Gourret *et al.*, 1992), respectively, using a kit from Pharmacia Biotech. An aliquot of the tracer cDNA was ligated to the *EcoRI/NotI* adaptor according to the kit's instructions and inserted into the

*Eco*RI site of λ gt10 and plated on non-permissive *Escherichia coli* strain C600 Hfl⁻ using Gigapack III packaging extract (Stratagene). The titre of the library was estimated at 1.7×10^7 pfu/ μ g of λ gt10 DNA.

A 200 ng portion of T and D cDNA (0.5 to 1 pmol of ends) was ligated to 280 pmol of the T (AATTCGC-CATGGATCTAGACC / pGGTCTAGATCCATG) and D (ATCAGTGATCGGCATGAGCTCG / pCGAGCT-CATGCCGA) specific linkers. A 50 ng portion of T and D DNA was then amplified for 12 cycles of PCR for the T (using the top T primer) and 30 cycles for the D using 50 nM Bio-11-dUTP (Sigma) and the top T primer. A 150 ng portion of amplified T was then subtracted with a 20-fold excess of biotinylated D for 24 h at 68 °C. The biotinylated DNA was then removed by the streptavidin/phenol extraction method. The subtraction step was repeated 2 times under the same conditions. 1/6 of the total subtracted tracer-driver (T-D) DNA was then amplified for 35 cycles using the T primer and the result was quantified on a gel. We chose to not directly clone the subtracted product as done by Rubinelli *et al.* (1998) but to use it as a probe to screen the microspore cDNA library in order to compensate the representational bias introduced by PCR.

Differential screening

A 20 ng portion of amplified subtraction product (T-D) and unsubtracted T cDNA were labelled at high specific activity with [³²P] dCTP with an oligolabelling kit (Pharmacia Biotech) (Feinberg and Vogelstein, 1985) and hybridized on duplicate filters (colony/plaque Screen filters, NEN Research Products, DuPont) containing approximately 20 000 plaques of the microspore cDNA library at 65 °C in 6× SSC for 24 h. The filters were washed in three steps of 30 min in 2×, 1× and 0.1× SSC with 0.1% SDS at 65 °C.

Only the clones showing a higher signal when hybridized with the T-D cDNA probe than with the T cDNA probe were selected and isolated. The accuracy of this choice was confirmed by a second round of hybridization realised in the same conditions on filters containing only the selected clones. Positive cDNA clones were purified and λ DNA was extracted as previously described (Albani *et al.*, 1990). cDNA inserts were isolated after *Not*I or *Eco*RI digestion and subcloned in pBluescript SK⁻ plasmid (Stratagene) digested by *Not*I or *Eco*RI.

Northern blot hybridization

A 10 μ g portion of denatured total RNA was loaded in each lane of a 1.5% agarose gel containing formaldehyde and transferred onto Hybond-N membrane (Amersham) according to the manufacturer's protocol. Prehybridization and hybridization were carried out in a buffer containing 5× SSPE, 5× Denhardt's solution, 100 μ g/ml sonicated salmon sperm DNA, 1% SDS and 50% deionized formamide at 42 °C for 6 h and 20 h, respectively. The selected cDNA fragments were gel-purified, labelled with [³²P] dCTP and used as probes for hybridization. The filters were washed at 65 °C in three 15 min steps in 0.1% SDS and decreasing salt concentrations (5×, 2× and 0.1× SSPE).

Genomic DNA library screening

350 000 recombinant clones (average size 15 kb) (three *B. napus* genome equivalents) of a genomic DNA library of *B. napus* cv. Bridger in bacteriophage λ EMBL-3 (Clontech, USA) were plated with *E. coli* LE392 as host. The entire M3 cDNA was used as a [³²P] dCTP-labelled probe for screening. Hybridization and washing were carried out with standard techniques (Sambrook *et al.*, 1989).

Determination of the 5' end of mRNA

The method was based on PCR using the 5'-AmplifINDER RACE kit from Clontech.

Two antisense primers were designed from the *BnM3.4* coding sequence: P1, 5'-TGATGACTCTAGT TCTGTTGCTGTG-3' and P2, 5'-TGGAAATTCGTTCT GTTGCTGTGACTTTGGATGT-3', and we optimized the annealing temperature at 55°C for the step of PCR amplification. The experiment was performed with 2 μ g of mRNA extracted from *B. napus* microspores, from *B. napus* floral buds and from *B. napus* leaves for the negative controls. Thereafter PCR products were digested by *Eco*RI and cloned into pBluescript SK⁻ for sequencing.

Genomic DNA isolation and Southern blot hybridization

Genomic DNA was purified from seedling leaves using a standard procedure (Dellaporta *et al.*, 1983) followed by standard CsCl gradient centrifugation. Southern analysis was performed by digestion of 10 μ g of genomic DNA, electrophoresis of DNA

fragments on a 0.8% agarose gel and blotting onto Hybond-N membrane using standard methods (Sambrook *et al.*, 1989). The M3 cDNA fragment was gel-purified and labelled with [^{32} P] dCTP as described previously. A Southern blot was hybridized overnight at 65 °C in 6× SSC and washed at 65 °C in three 15 min steps in 0.1% SDS and decreasing salt concentrations (6×, 2× and 0.1× SSC).

Plasmid construction

Recombinant DNA techniques were carried out as described by Sambrook *et al.* (1989) to construct a transcriptional fusion bringing the expression of the *uidA* (commonly named *gus*) reporter gene under the control of the *BnM3.4* promoter in binary vector.

A 'promoterless' *gus* gene cassette, containing the *gus* coding sequence (Jefferson *et al.*, 1986) and the nopaline synthase polyadenylation site (*nos-ter*; Bevan *et al.*, 1983) was cloned into pBluescript SK– (Stratagene, USA), leading to pAF1opt. A 2056 bp *Bam*HI–*Nsp*V fragment (Figure 3) from the genomic clone *BnM3.4* was cloned into *Bam*HI–*Cla*I sites of the plasmid pAF1opt, generating pJD51. The chimaeric gene was then excised from pJD51 and introduced into the binary plasmid pEC2 (Cartea *et al.*, 1998) in inverse orientation with respect to the Basta resistance gene, generating pJD101. The promoterless *gus* gene cassette was also transferred into pEC2 to generate the negative control binary plasmid pAF100. The binary plasmids pAF100 and pJD101 were introduced into *Agrobacterium tumefaciens* strain C58C1 (Koncz and Schell, 1986) by electroporation (Nagel *et al.*, 1990; Singh *et al.*, 1993). The recombinant genes were introduced into *A. thaliana* by *in planta* infiltration (Bechtold *et al.*, 1993).

In situ hybridization

The *BnM3.4* coding sequence was cloned in both orientation in pGEM-3Zf(+) (pJD6 and 7). After *Sma*I linearization, these two plasmids were used to synthesize digoxigenin-11-rUTP-labelled probes using the Riboprobe Combination System T7 kit from Promega.

B. napus floral buds were fixed in 4% formaldehyde, embedded in wax, and 8 µm-sections were prepared for *in situ* hybridization according to Jackson (1991).

Full-length RNA probes were alkaline-hydrolysed to 150 nt fragments and hybridized to sections at a concentration of 0.5 ng/ml per kb of probe. Hybridization was carried out in 210 µl for a sandwich of

two slides at 50 °C in 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 0.3 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA, 1 mg/ml yeast transfer RNA. Slides were washed in several baths of 0.2× SSC at 55 °C for 1 h, followed by two rinses of 5 min each with 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (NTE buffer), and treated with 20 mg/ml RNase A in this buffer at 37 °C for 30 min. The slides were then washed again in NTE buffer and 0.2× SSC as described above, and finally washed in PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7) for 5 min. Immunological detection of the hybridized probe was carried out as described in the Boehringer digoxigenin-nucleic acid detection kit with some modifications. Slides were incubated with gentle agitation for 1 h in 0.5% blocking agent (Boehringer) in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) followed by 1 h in 1% bovine serum albumin, 0.3% Triton X-100 in buffer 1 (buffer 2). This was followed by a 1 h incubation in dilute antibody conjugate (Boehringer) (1:1250) in buffer 2 and 4 washes of 15 min each in buffer 2. Slides were briefly washed in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂ and incubated for 12–48 h in 0.34 mg/ml nitroblue tetrazolium salt and 0.175 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂. The colour reaction was stopped with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and sections were passed through an ethanol series before mounting in mounting medium from Sigma.

Histochemical GUS assays

Histochemical assays for GUS activity were conducted according to the protocol described previously by Jefferson *et al.* (1987) with some modifications. Fresh tissue samples were fixed in a solution containing chloroform, 95% ethanol and water in proportions 3:6:1 and 0.1% Triton X-100 under vacuum (–93.3 kPa) for 1 min. Then, tissue samples were washed twice in 50 mM potassium phosphate buffer, pH 7.0. GUS staining was performed by vacuum infiltration (3× 10 min at –93.3 kPa) in the GUS stain solution (Jefferson *et al.*, 1987) and by incubating at 37 °C overnight. The plant material was then cleared by rinsing with 70% ethanol and the samples were examined and photographed under a Diaplan type 307-148.002 microscope (Leitz, Wetzlar, Germany).

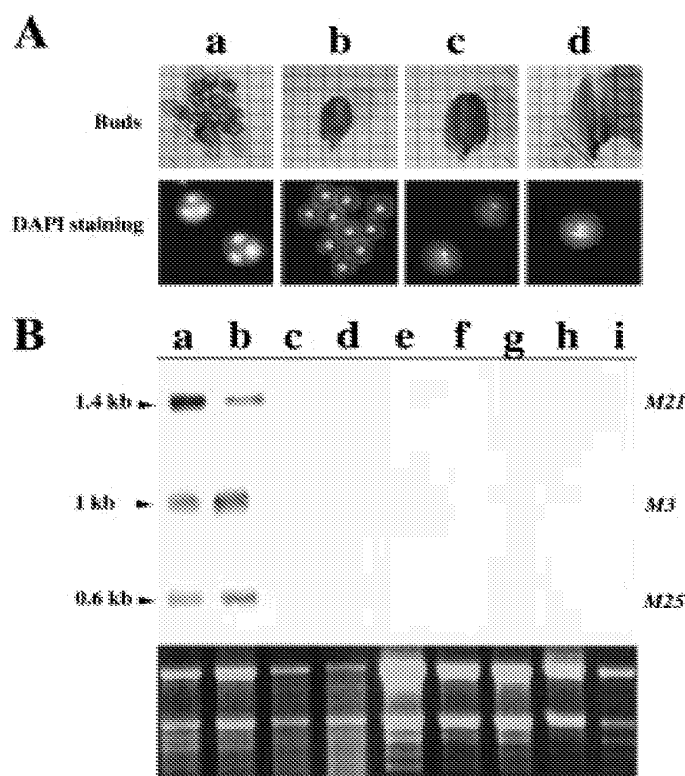


Figure 1. Correlation between the size of rapeseed flower buds and microgametophytic stage of development. Expression pattern of the M21, M3 and M25 clones isolated by subtraction and differential screening of the microspore cDNA library. A. DAPI staining of the four different developmental stages during male gametogenesis (in the lower part) and the corresponding buds (in the upper part). a, DAPI-stained tetrads and earlier stages (not shown) contained in buds of length strictly below 2 mm; b, microspores contained in buds of 2–3 mm; c, bicellular pollen contained in buds of 3–4 mm; d, tricellular pollen contained in buds of 4.5 mm and more. Each square is 1 mm $\times$ 1 mm. B. Northern blot experiments hybridized with M21, M3 and M25 cDNA clones as probes. Total RNA (10 μ g) from (a) meiocyte/tetrads, (b) microspores, (c) young (binucleate) and (d) mature (trinucleate) pollen, (e) sepals, (f) petals, (g) wild type and (h) CMS buds, and (i) *in vitro* grown seedlings were loaded on the gel. The picture of the corresponding ethidium bromide stained gel is presented underneath.

The pistil length was used as the unique criterion for identification of the stages of microsporocyte development in *A. thaliana*. Based on results of Bowman (1994), the correlation between the pistil length (pl) and the stages of microsporocyte development was further studied (D. Vezon, personal communication) and we found that a pl of 0.3–0.4 mm corresponds to meiosis; pl 0.5 mm corresponds to the end of meiosis and tetrad microspores; pl 0.75 corresponds to free microspores; pl 1.25 mm corresponds to the first pollen mitosis and bicellular pollen; pl 1.50 mm corresponds to the second pollen mitosis and tricellular pollen.

Results

Isolation of B. napus cDNAs encoding sequences expressed early during pollen development

Cytological studies (Scott *et al.*, 1991) have shown that the stages of male gametogenesis within the anther are highly correlated to bud length and that gametogenesis is synchronised in all anthers of one flower bud.

The cytological studies allowed us to establish that, in the growth conditions we used, rapeseed buds of length strictly below 2 mm contained mainly meiocytes and tetrads, 2 to 3 mm long buds contained

M3									80
M3.21	TCTTGCTATG	ATTTCCTCA	TAAGATGTGT	CACATCCAAA	GTACAGCAA	CAGAACTAGA	GTCATCAACT	AACCAAGAGC	
M3									160
M3.21	TCTTCCTATC	GCGGCACTTG	CCTCGCTTTC	ACCCCAAGCC	ACATTGGCCG	TTCTGTGGCT	CCGGAAGAGC	CTTCCCTGCA	
M3									240
M3.21	GGCCACTTCC	GACCACTCC	GTTCCATCTG	CCACAGGAAG	TCACCAGATG	CTGTGCCGAC	AAGAAGGAGG	TAGGTACATG	
M3									320
M3.21	TTTGTATGAT	ATCGTTGAGA	CTTCTTCAC	CAGGAAGCC	GTTATTGGAT	CGGAATGTTG	CGCCGCGATC	AAGAAGATGA	
M3									400
M3.21	ACAAAGATTG	TGAGAAGACC	GTCTTGGAT	CTTTCCATGA	CCCTTCTTG	ACCGGCTATG	TCAAGCTACA	TTGCTCCACC	
M3									480
M3.21	GTGTGTGGAT	CTACTTCACC	TCCTCCTTCA	CAGGCTCCTT	TACATGCTCC	TTCTTCACAG	GCTCCTTCAC	ATGCTCCTTC	
M3									560
M3.21	ACATGCTCCT	TCACAGGCTC	CTTTAAATGC	TCTTTTAAAT	GCTCCTTTAC	ATGCTCCTTT	ACATGCTCCT	TCACAGGCC	
M3									640
M3.21	CTTCACAGGC	CCCTTCACAG	GCCCCTTTAC	ATGCTCCTTT	ACTGCCCCCT	TCGCAGGCTC	CTTCACCGGC	TCAGTGA-TT	
M3									720
M3.21	TAGCTATTTG	ATAGAATTAC	TCAAGTAATG	ATGCCCTAGG	GAGTTTGAGT	TTTCTCGTG	TTTTAAAGTT	TGTGTATTAT	
M3									800
M3.21	TTTGAGAAAA	CCGTCTTTGG	ATTTTAACTT	CACTTTGATT	TTTTCCCTTA	TACAATTAA	ATTTAGAGTT	TACTTATTAA	
M3									841
M3.21	TTTTATAAAT	TAGATGGTAC	TAAGTTTTTA	TCATAATAAA	A				

Figure 2. Comparison of M3 and M3.21 cDNA sequences. Numbers at the right margin refer to the nucleotide position. Dashed lines indicate gaps and underlined sequences represent imperfect repeated motifs found in the M3 and M3.21 sequences. The stop codons are shown in bold type.

mainly microspores, 3 to 4 mm long buds contained mainly bicellular pollen grains and buds of 4.5 mm and more in length contained mature tricellular pollen grains (Figure 1A). RNAs were prepared from male gametophytes purified from buds at these four stages. To achieve this we had to develop a new technique (see Materials and methods) to lyse the microspores which have extremely resistant cell walls. To obtain a driver for the subtraction procedure, we isolated RNA from whole male-sterile flower buds (Ogu-INRA). These buds are identical to those of wild type except that male gametophyte development is arrested before the tetrad stage and consequently they do not contain any microspores (Gourret *et al.*, 1992).

Poly(A)⁺ RNAs from microspores were converted to cDNA, of which a fraction was used to construct a cDNA library. To generate probes enriched for microspore-specific transcripts, another fraction of the above microspore cDNA was then subtracted sev-

eral times by hybridization with cDNA made from male-sterile buds (see Materials and methods). We then screened the microspore cDNA library using subtracted and primary probes. After two rounds of such successive screens, we retained 32 potential candidates that showed increased hybridization to the subtracted probe with respect to the unsubtracted microspore cDNA probe.

The expression pattern of selected clones was compared in more detail by slot blot (data not shown) and by northern blot analyses with total RNA isolated from the four same developmental stages of male gametophyte as above, entire wild-type and male-sterile buds, sepals, petals and *in vitro* grown seedlings or leaves. These expression studies allowed us to classify these genes into four groups.

The first group consists of 13 clones whose expression is strictly confined to the male gametophyte and high in the microspore. The second group contains 13

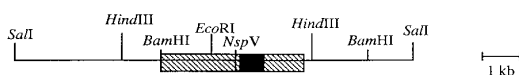


Figure 3. Analysis of the *BnM3.4* gene. Partial restriction map of the *BnM3.4* genomic clone. The striped box represents the sequenced region of the *BnM3.4* genomic clone; the black one the coding sequence of the *BnM3.4* gene. The two *SalI* sites border the 10 kb insert.

clones whose expression is high in the microspores but not restricted to the male gametophyte since they are also expressed in other sporophytic tissues. The third and fourth groups correspond respectively to 2 clones having a weak expression restricted to microspores and 4 clones showing weak expression in all tissues tested.

We characterized further three cDNAs belonging to the first group of genes expressed early and specifically during male gametophyte development. Northern blot analyses of these clones are shown in Figure 1B. The M21 gene detected a mRNA of 1.4 kb (encodes potentially a novel protein), which is expressed at the meiocyte and tetrad stages but its mRNA level remains high in the microspore. The M25 cDNA detected a 0.6 kb mRNA (encoding a putative SKP1-like protein (Connelly and Heiter, 1996; Krek, 1998)), mainly in the microspore but expression was also detected at meiocyte/tetrad stages and in bicellular pollen. The M3 cDNA detected a 1.0 kb mRNA in microspores but expression was also evident in meiocyte/tetrad stages. We have chosen to focus on this particular clone for further studies because extensive hybridization studies using a variety of sporophytic tissues (data not shown) suggested that the M3 mRNA is highly early stage microspore-specific.

Analysis of the nucleotide sequences of M3 and M3.21 cDNAs

The M3 cDNA has a length of 497 bp. It was smaller than the 1.0 kb mRNA detected on northern blots, so M3 is likely to be a partial cDNA. A second screening of the microspore cDNA library was performed using M3 as probe. A longer but still partial clone, named M3.21, of 674 bp was then isolated. The expression pattern of the M3.21 cDNA was checked by northern blot (data not shown) and appeared to be identical to the M3 pattern. Both cDNA clones, M3 and M3.21, were subcloned into pBluescript SK[−] and sequenced. Alignment of their nucleotide sequences shows that the M3 cDNA differs essentially from M3.21 by an

insertion of 99 bp constituted by three repeats of the same motif (Figure 2).

M3 cDNA and the corresponding BnM3.4 genomic clone: DNA sequence comparisons and analyses

The M3 cDNA was used as a probe to screen a *B. napus* genomic DNA library in order to isolate the corresponding gene. One of the isolated clones, BnM3.4, with an insert size of 10 kb, was selected for further characterization (Figure 3). A 2.9 kb sequence surrounding the *BnM3.4* gene was deposited in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number AF136223. Two putative in-frame translation start codons, separated by 114 bp and leading to 2 ORFs of 712 bp and 598 bp, were detected (respectively at 1971 and 2085 nucleotides from the start of the sequence). Comparison of the M3 cDNA sequence with the sequence of the shortest ORF of the genomic clone revealed 1 difference out of 293 bp of aligned coding sequence and 7 differences out of 203 bp of aligned 3'-downstream region. These sequence differences between genomic and cDNA clones were repeatedly found in independent sequencing experiments and might be due to allelic variations between the different *B. napus* varieties used in the construction of the cDNA and genomic libraries. No intron was detected in the region covered by the cDNA and no intron/exon junctions were found in the rest of the ORF using a splice site prediction program (Hebsgaard *et al.*, 1996). A putative polyadenylation signal (Joshi, 1987b) was found 197 bp downstream of the stop codon. Because we are ultimately interested in the regulation of pollen and anther-specific genes, we first determined by 5' RACE which of the two potential start codons in M3/M3.21 are used exclusively or preferentially. Sequences of 12 PCR fragments revealed two groups of products. The first group contained seven sequences identical to the *BnM3.4* gene (PCR1 in Figure 4). The longest PCR product showed that the transcription start of the *BnM3.4* gene is a G (underlined on Figure 4), localized between the two potential translation starts. Primer extension experiments (data not shown) indicated approximately the same nucleotide for the transcriptional start site. The length between the transcriptional start site and the second ATG (64 bp), is in agreement with the predominant length of leader sequences (40–80 nucleotides) in plant genes (Joshi, 1987a). Moreover, the context of the second ATG agrees well with the consensus sequence (AAorCAAUGGC) proposed for

BnM3.4	-21	AACCCCTAACG	CCTTACCACT	CGATAACCAT	CAAAACTTTT	CTTCTCGTTT	29
PCR1				.GATAACCAT	CAAAACTTTT	CTTCTCGTTT	
PCR2		.	ACT	CAATAACCAT	CAAAACTTTT	CTTCTCGTTT	
BnM3.4		CGCTAACTCA	AGGCTTCGAA	AAGTAAAAA	AACAATGAAG	AATGTCACAC	79
PCR1		CGCTAACTCA	AGGCTTCGAA	AAGTAAAAA	AACAATGAAG	AATATCACAC	
PCR2		CGCAAACTCA	AAGCTTCAAA	AAGT---AAG	AACAATGAAG	AATGTCTCAC	
BnM3.4		TGTTCTTGC	TATGATCCTC	TTCTTAAGCT	GTGTCACATC	CAAAGTTACA	129
PCR1		TGTTCTTGC	TATGATCCTC	TTCTTAAGCT	GTGTCACATC	CAAAGTTACA	
PCR2		TGTTCTTGC	TATGATCCTC	TTCTTAAGCT	GTGTCACATC	CAAAGTCACA	
BnM3.4		GCAACAGAAC	TAGAGTCATC	AACTAACCAA	GAGCTCTTCC		169
PCR1		GCAACAGAAC					
PCR2		GCAACAGAAC					

Figure 4. Alignment of the *BnM3.4* gene sequence and the longest representatives of the two types of products PCR1 and PCR2 from the 5' RACE PCR on mRNA from *B. napus* microspores. Dashed lines indicating gaps and nucleotide differences are reported in bold type in PCR2. The transcription start site (C) is underlined (in *BnM3.4* and PCR1) and the ATG codon is in bold type.

dicotyledon nuclear genes by Joshi *et al.* (1997). A clear TATA box sequence (TATATATA) was identified 31 nucleotides upstream of the start of transcription (Messing *et al.*, 1983). The second group of 5' RACE PCR products (PCR2 in Figure 4) presents several nucleotide differences with respect to the first group. The detection of this second group proves that a second gene closely related to *BnM3.4* exists and is expressed in the microspore at approximately the same level as *BnM3.4*. It should not correspond to allele variation of *BnM3.4* because cv. Brutor used to make the cDNA library and the 5' RACE experiments is an inbred line. This gene may correspond to the M3.21 cDNA but M3.21 is too short at its 5' end to enable us to compare it to the PCR2 sequences and determine whether they come from the M3.21 type mRNAs. To ascertain this, a Southern blot analysis was performed on total DNA extracted from *B. napus* cv. Brutor and digested by *Bam*HI, *Eco*RI and *Hind*III. The DNA gel blot was hybridized with ³²P-labelled M3 cDNA at high stringency. As shown in Figure 5, in addition to the 5 kb *Hind*III and 5.5 kb *Bam*HI fragments corresponding to the *BnM3.4* genomic clone, the *B. napus* genome contained other fragments which were clearly recognized by the M3 cDNA probe, consistent with the idea of at least one other expressed homologue in *B. napus*. These homologous genes will be called the *BnM3* family.

Characteristics of the putative *BnM3.4* protein

The *BnM3.4* gene encodes a putative 218 amino acid protein with a molecular mass of 23.4 kDa and a predicted pI of 8.8. The predicted *BnM3.4* protein shows limited similarity with two potential *A. thaliana*

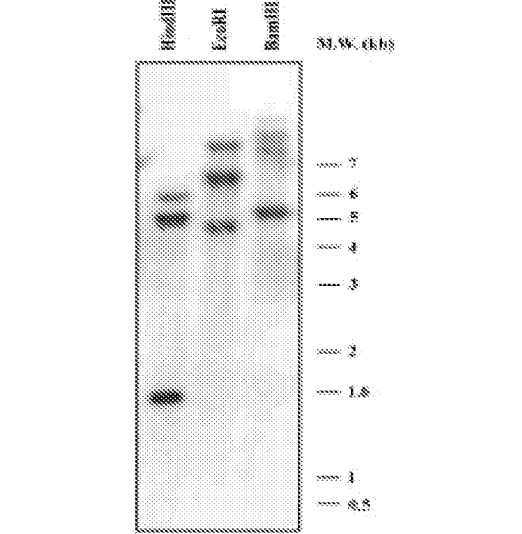


Figure 5. Southern blot analysis of genomic DNA from *B. napus* (cv. Brutor). A 10 µg portion of genomic DNA was digested with *Bam*HI, *Eco*RI and *Hind*III. Hybridization was performed with ³²P-labelled M3 cDNA as probe. DNA size standards (in kb) are shown to the right of the figure.

products of genomic clones MKP11 (accession number AB005238) and K19P17 (AB007644). The N-terminal 152 aa of *BnM3.4* is 55% identical (69% similarity) to MKP11, while the carboxyl portion of *BnM3.4* is 45% identical (63% similarity) to K19P17. These two translated sequences do not correspond to two exons of the same gene since they are separated

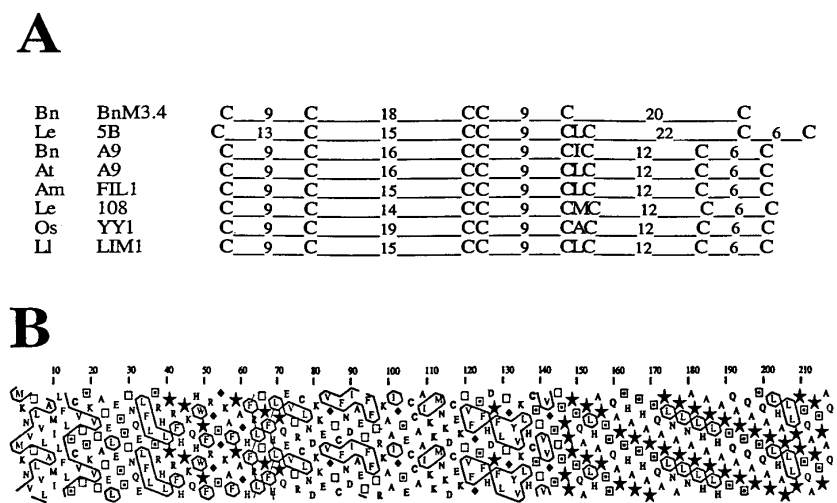


Figure 6. BnM3.4 protein characteristics. A. Cysteine pattern alignment of the BnM3.4 protein with sequences of seven stamen-specific genes. The number of amino acids between cysteine residues is given, but the spacing is not proportional. Le 5B, *Lycopersicon esculentum* TomA5B (Aguirre and Smith, 1993); Bn A9, *Brassica napus* A9; At A9, *Arabidopsis thaliana* A9 (Paul *et al.*, 1992); Am FIL1, *Antirrhinum majus* FIL1 (Nacken *et al.*, 1991); Le 108, *Lycopersicon esculentum* 108 (Chen and Smith, 1993); Os YY1, *Oryza sativa* YY1 (Hihara *et al.*, 1996); Li LIM1, *Lilium longiflorum* LIM1 (Kobayashi *et al.*, 1994). The amino acid sequences were deduced from DNA sequences. B. HCA (Hydrophobic Cluster Analysis) representation of the BnM3.4 protein. Amino acids are represented with one-letter symbols except for: proline (stars); glycine (diamonds); threonine (open squares); and serine (squares with dot). Hydrophobic clusters are endorsed by a black line.

by 16 Mb on the chromosome. The functions of these two putative *A. thaliana* gene products are not known.

The putative BnM3.4 protein, however, has three recognizable structural domains. The residues from 1 to 23 are hydrophobic, characteristic of a signal peptide, and a cleavage site between the alanine at position 23 and the threonine at position 24 can be predicted according to the rules of von Heijne (Nielsen *et al.*, 1997). From these features, the protein may be secreted and this N-segment may help the protein to cross or to interact with the membrane. The central part of the protein (residues 25–143) is hydrophilic. The spacing of six particular cysteines in this segment resembles that found in various stamen-specific proteins: TomA5B (Aguirre and Smith, 1993), FIL1 (Nacken *et al.*, 1991), A9 (Paul *et al.*, 1992), 108 (Chen and Smith, 1993), YY1 (Hihara *et al.*, 1996) and LIM1 (Kobayashi *et al.*, 1994). The possible relationship among these cysteine-rich proteins and the BnM3.4 protein is best illustrated by showing only their pattern of cysteine residues (Figure 6A). Although the BnM3.4 protein presents a shorter pattern than the others, the position and spacing of the cysteine residues are very similar.

The C-terminal domain consists of 14 repeats related to the motif P-[SL]-[HNQ]-A. Analysis of this domain by the Hydrophobic Cluster Analysis program (available at: www.lmcp.jussieu.fr/~soyer/www_hca/hca-form.html) suggests that the proline residues in the motifs could form the backbone of a rigid helical structure (Figure 6B).

In situ analysis of the expression of the BnM3 genes family

The pattern of *BnM3* expression during the four stages of gametogenesis (see above) was examined in bud tissues of *B. napus* by *in situ* hybridization using the *BnM3.4* antisense transcript as a probe. As at least two homologous genes are present in the *B. napus* genome, the hybridization pattern may reflect the expression pattern of (at least) these two genes. The *BnM3* transcripts were not detectable in anthers with pollen mother cells undergoing meiosis (data not shown), nor in tetrads (Figure 7A), nor in bicellular pollen (Figure 7G), nor in tricellular pollen (Figure 7I). However, at the developmental stage where rapeseed buds are 2.5 mm, a strong hybridization signal was detected in the microspore (Figure 7C). At high magnification

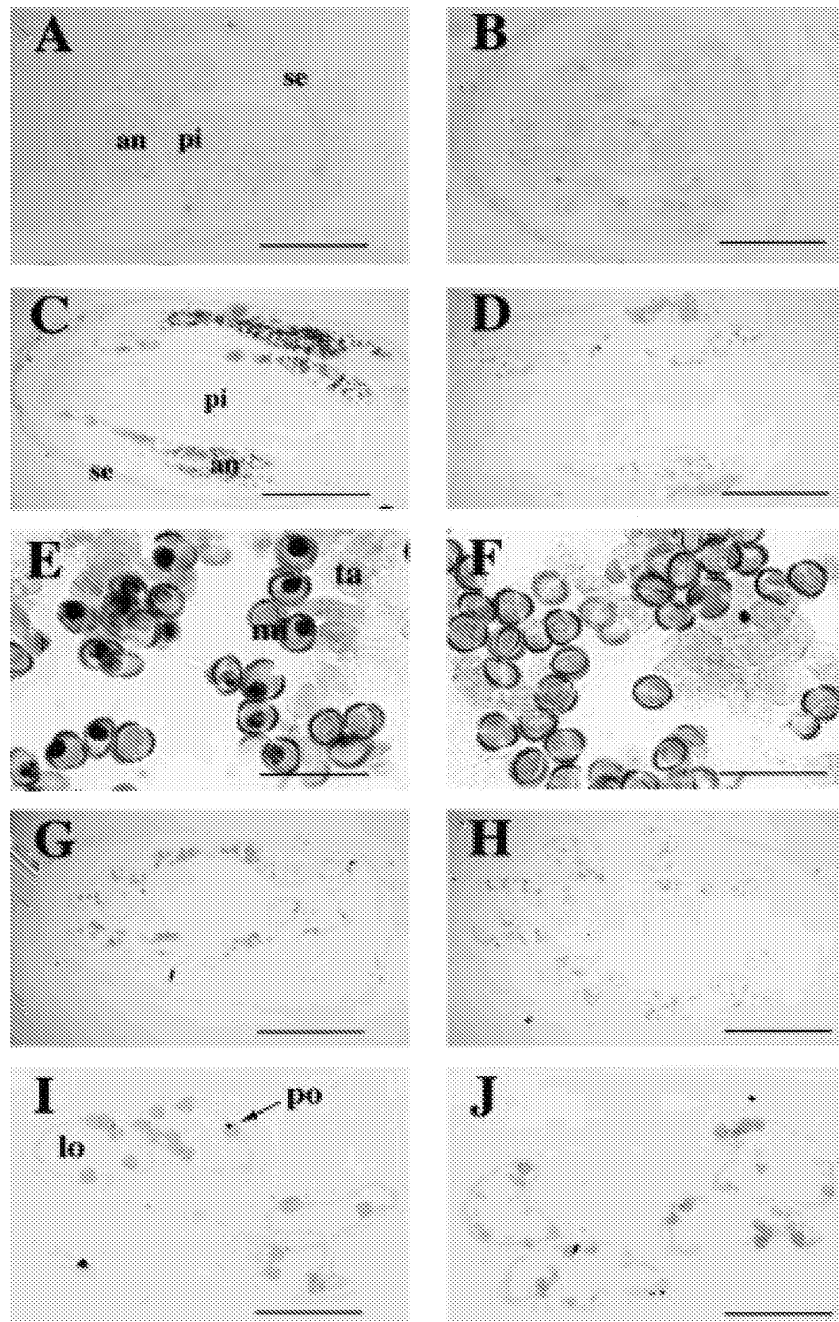


Figure 7. *BnM3.4* expression in floral buds of *B. napus*. *In situ* hybridization of DIG-labelled antisense (A, C, E, G and I) or sense (B, D, F, H and J) *BnM3.4* RNA probe to *B. napus* floral bud sections. A and B. Transverse sections of 1.5 mm long buds containing tetrads (bar = 4 μ m). C and D. Longitudinal sections of 2.5 mm long buds containing microspores (bar = 10 μ m). E and F. Magnification of anthers from transverse sections of 2.5 mm long buds (bar = 0.8 μ m). G and H. Transverse sections of 3.5 mm long buds containing bicellular pollen (bar = 10 μ m). I and J. Transverse sections of anthers from 4.5 mm long buds containing tricellular pollen (bar = 4 μ m). Abbreviations: an, anther; lo, locule; mi, microspore; pi, pistil; po, mature pollen; se, sepal; ta, tapetal cell.

(Figure 7E), the *BnM3* transcripts are clearly visible by a deep staining inside the microspores. No hybridization signal was detected in any male gametophyte tissue at any stage of development with the sense probe (Figure 7B, D, F, H, J). The lack of hybridization of some microspores with the antisense probe is probably an artefact caused by a partial section of the pollen grain or a loss of cytoplasm during the pretreatment of slides. Both the sense and the antisense probes allowed us to detect a weak hybridization signal in tapetal cells that could be considered as a background signal not specific to the *BnM3.4* transcripts.

The BnM3.4 promoter: regions of homology and functional analysis

A detailed analysis of the *BnM3.4* promoter sequence revealed the presence of several regions having possible regulatory functions. Two 14 bp repeats (tttaaaagtgaac) are located from -1852 to -1780 from the transcription start. A 14 bp palindromic sequence (ttagaatattctaa) is located at -307 while another 12 bp palindrome (ttttaattaaaa) starts at -213. These motifs have not been detected in any other pollen-specific gene. However, the motif 'tcattt', located between -593 and -517 and the reverse motif 'aatga', located at -1566 in *BnM3.4* upstream regions were already described for other pollen-specific genes like *NTP303* (Weterings *et al.*, 1995), *NTM19* (Oldenhof *et al.*, 1996) and *Bp10* (Albani *et al.*, 1992). A 2 kb region of this 5'-flanking sequence, containing these different regions was transcriptionally fused with the reporter *gus* coding sequence. The resulting construction (pJD101, see Materials and methods) was introduced into *A. thaliana*. This species was chosen for these experiments because it belongs to the same botanical family as *B. napus*, but it can be more easily transformed and has a shorter life cycle. GUS staining was first performed on the inflorescences of fifteen independent transgenic *A. thaliana* T₁ lines (JD101 series) transformed with this gene. They all showed qualitatively the same expression patterns in the course of gametophyte development (not shown). The progeny (T₂) of T₁ plants with insertion of the transgene(s)

presumably at a single locus was chosen for further analysis. The results of histochemical analysis of *gus* expression for individual hemizygous and homozygous progeny of one of them, JD101.42, are shown in Figure 8. This transformant presents one insertion according to the genetic test (275 phosphotricine resistant plants out of 380 seedlings; $\chi^2 = 1.40$; $P < 0.05$).

In all the transgenic JD101 plants and their progeny studied, no GUS activity was detected in flower organs other than the stamen as shown in Figure 8A for one hemizygote progeny of the JD101.42 plant. GUS activity was not detected in any organ of seedling (Figure 8I) or older plants (not shown), or in siliques (Figure 8J). In comparison, plants transformed with the control construction pAF100 (*gus* without promoter) showed no GUS activity (Figure 8A, inset). The presence of 50% of blue pollen and 50% of unstained pollen in anthers of the hemizygous JD101.42 plant (Figure 8H) confirms that the *BnM3.4* promoter is only activated in gametophytic cells i.e. only the transgenic pollen shows GUS activity.

The temporal pattern of *gus* expression was determined with homozygous JD101 progeny with the aim of precisely determining the developmental stage where the reporter gene is activated. At the tetrad stage of male gametophyte, no GUS activity was detected (Figure 8B); it only appears at a high level at the beginning of the microspore stage (Figure 8C). As shown in Figure 8E, high levels of GUS activity were detected in pollen. There seems to be a low level of the GUS product in the locule which may be the result of diffusion, but no activity was present in the sporophytic tissues of the anther such as the epidermis, the endothecium and the tapetum. The GUS expression level stays constant until the mature tricellular pollen stage (Figure 8D, 8F and 8G) but it is difficult to know if this is due to the longevity of the GUS protein or transgene activity in *Arabidopsis*.

At least for the first stages of male gametophyte development, the reporter gene has the same expression pattern as intact genes.

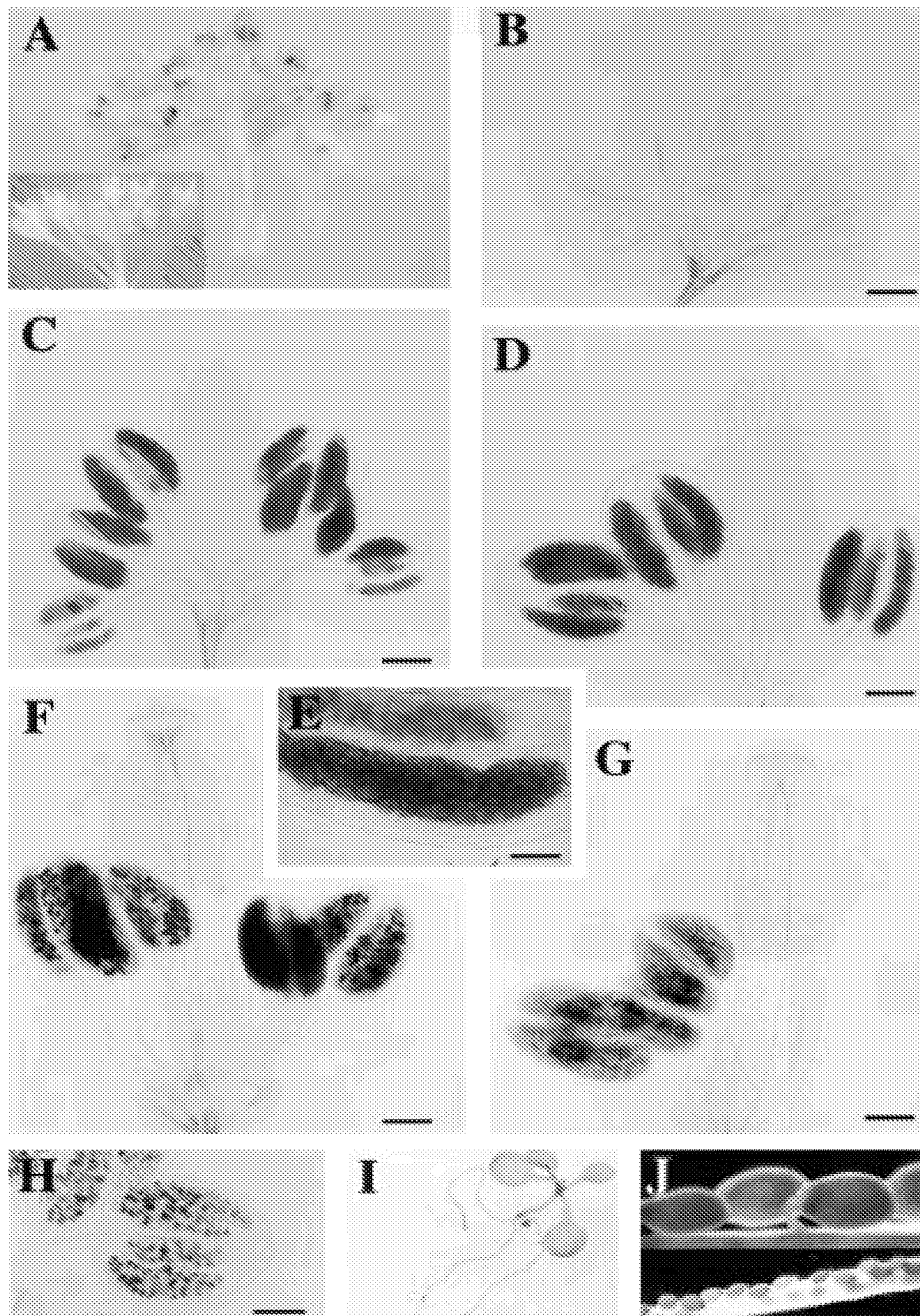


Figure 8. Histochemical analysis of *gus* expression in transgenic *A. thaliana* JD101 plants. The following plant materials from homozygous and hemizygous JD101.42 transgenic plants and from a AF100 transgenic plant were examined for GUS activity as described in Materials and methods. A. Inflorescence from a 5-week old homozygous pJD101 plant and (in the inset) the inflorescence from a 5-week old AF100 plant (negative control plant). B–G. Developmental series of buds from the stained inflorescence presented above. Sepals and petals were removed from the bud in order to measure the pistil length more easily and to improve the visualization of GUS in anthers. The stages of male gametophyte development were determined according to the pistil length as described in Materials and methods. B, tetrad stage; C, early microspore stage; D, end of microspore stage; E, locule of one of the anthers of the bud shown in D, magnified 6× relative to Figure D (bar represents 27 μm); F, bicellular pollen. g, tricellular pollen in anthers at anthesis. In B, C, D, F and G, bars represent 90 μm. H. Anther of a hemizygous plant, at the microspore stage (bar represents 70 μm). I. Ten-day old seedling. J. Open siliques and seeds at two different magnifications.

Discussion

This paper describes a method in order to isolate cDNAs from rapeseed that are preferentially expressed during early male gametophyte development. Expression of several of these genes appears to be highly specific to microspores and we have analysed one of these, called *BnM3.4*, in detail. This gene corresponds to the M3 cDNA isolated, but another cDNA was isolated from the microspore cDNA library using M3 as a probe. This longer cDNA (M3.21) is homologous to M3 except that it shows a deletion of 99 bp in the putative coding region. As these two cDNA cross-hybridize in the hybridization conditions used, we can conclude that at least two genes (the *BnM3* family=*BnM3.4* + related gene(s)) are present in the *B. napus* genome. This is also supported by the results of 5' RACE and Southern blot experiments.

The expression of these genes was studied both by northern blot and *in situ* hybridization. By northern blot analysis, the transcripts were observed mainly in microspores and also in total RNA extracted from buds of length strictly below 2 mm, that contain a majority of tetrads and meiocytes. The developmental synchrony between all the male gametophytes of an anther is not perfect and some free microspores already appear among tetrads in buds below 2 mm, so the hybridization of M3 probe to RNAs from pollen extracted from buds below 2 mm may be due to the early presence of microspores in these buds. This hypothesis was confirmed by *in situ* hybridization experiments. In individual 1.5 mm long buds, where no microspores were found, no signal was detected. On the other hand, in 2.5 mm long buds, where microspores are the only developmental stage present, a strong signal was detected. In later gametogenesis stages, no further signal was detected. According to these results, we can conclude that the homologous genes of *BnM3* family are expressed specifically in the microspore of *B. napus*.

Like the other early-expressed gene isolated from tobacco, *NTM19* (Oldenhof *et al.*, 1996), *BnM3.4* does not show any homology to known genes. Although the deduced *BnM3.4* protein does not show homology with the deduced *NTM19* protein, they are both proline-rich proteins and contain a putative signal peptide. We can only speculate on the function and cellular localisation of the *BnM3.4* protein with the help of prediction programs. Several stamen proteins of unknown function contain similar proline-rich domains (Chen *et al.*, 1993) that, despite differences in amino-acid composition, are structured in a very similar way. The role of the proline-rich motifs is not known, but one may speculate that they serve as some kind of anchoring structures in interaction with either components of the cytoskeleton or with structural components of the cell wall (Woessner *et al.*, 1994; Kröger *et al.*, 1996).

The comparison analysis of the *NTM19* and the *BnM3.4* promoter regions did not show any clear common domains which could indicate a role in the specificity of expression of these genes. Only the box 'tcattt' and its reverse sequence, 'aatga', which has already been shown to be important for pollen-specific expression are present in both promoters. Even if the number and the precise location of these motifs are not conserved between the different promoter regions described (tobacco for *NTM19* and *NTP303*, rapeseed for *Bp10*), functional analysis using deletion and targeted mutagenesis experiments in microprojectile-mediated transient expression assays (Weterings *et al.*, 1995) showed clearly that one of these elements of the *NTP303* promoter constitutes a positive *cis*-regulatory element and functions by specifically enhancing transcription in pollen. This is the reason why we kept this promoter region of 2 kb for the heterologous expression experiments. Both analysis of more microspore-specific genes and promoter deletions using a reporter gene will be necessary to evaluate the influence of the different motifs in the spacial and temporal regulation of transcription of these genes.

The histochemical analysis of *gus* expression driven by the *BnM3.4* promoter (pJD101 construction) in transgenic *A. thaliana* plants showed a high level of GUS activity in male gametophytes but not in other floral and seedling tissues. Furthermore, GUS activity appeared at the microspore stage and stayed at a constant level during pollen development until pollen maturity. GUS being a very stable protein, the detection of GUS activity allowed us to define precisely the start point of *BnM3.4* promoter activity in *A. thaliana* but not its stop point. The spatial and temporal pattern of *gus* expression driven by the *BnM3.4* promoter in *A. thaliana* is in accordance with the established expression pattern of the gene in *B. napus*. These results suggest the presence of *trans*-acting factors in *A. thaliana* able to recognise and interact with the *cis*-regulatory elements of *BnM3.4* in the same manner as in *B. napus*.

Several of the genes isolated by this procedure belong to gene families (*BnM3.4*, *SKP1*-like genes) as seen with other transcriptional approaches (Twell, 1994). This strategy is complementary to the mutagenesis being undertaken in *A. thaliana* (Feldmann *et al.*, 1997; Bonhomme *et al.*, 1999). We now plan to compare the 5'-flanking regions of the other microspore-specific genes we have isolated and to try *BnM3.4* promoter deletion analysis in order to find *cis*-regulatory elements required for microspore-specific expression.

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Application	Document	Mailroom Date	Attorney Docket No.
14491197	CTNF	01/12/2017	DAS-P0129-04-US-E
	892	01/12/2017	DAS-P0129-04-US-E

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Customer No.: 107540 }
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Group: 1663 }
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Confirmation No.: 3336 }
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Application No.: 14/491,197 }
 }
Invention: **NOVEL HERBICIDE** }
 RESISTANCE GENES }
 }
Inventors: Wright, Terry R., et al. }
 }
Filed: September 19, 2014 }
 }
Attorney Docket: 14764-245772 }
 }
Examiner: KRUSE, DAVID H. }

FILED ELECTRONICALLY:
April 12, 2017

RESPONSE AND AMENDMENT UNDER 37 C.F.R. § 1.111

Mail Stop Amendment
Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

In response to the Office Action mailed January 12, 2017, Applicant respectfully requests entry of the following amendments and consideration of the accompanying remarks.

Applicant believes that no fees are required with this response. However, if any fees are required, the Commissioner is hereby authorized to charge those fees to the account of

U.S. Application Serial No. 14/491,197

Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-245772.

Amendments to the Specification begin on page 3 of this paper.

Amendments to the Claims begin on page 4 of this paper.

Remarks begin on page 9 of this paper.

AMENDMENT TO THE SPECIFICATION

Please replace paragraph 00358 on page 105 with the following modified paragraph.

[00358] 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.

Table 26.b

Construct	Line Number	AAD-12 PTU	AAD-12 Invader	AAD-12 ng/ml
pDAB724	1	+	+	79.89
pDAB724	2	+	+	17.35
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

AMENDMENTS TO THE CLAIMS

A complete listing of the pending claims is provided pursuant to 37 C.F.R. § 1.121(c)(1). Please amend claims 1, 3-5, 7-11, 17, 19-22, 24, and 26 as follows. Please cancel claim 25 without prejudice.

In the Claims

1. (Currently Amended) An expression cassette for expression in a plant cell, comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, ~~that is functional in a plant cell~~, wherein said polynucleotide encodes a protein that catalyzes degradation of a herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.

2. (Original) The expression cassette of claim 1, wherein said polynucleotide comprises plant codons for expression in said plant cell.

3. (Currently Amended) The expression cassette of claim 1, wherein said plant promoter or said plant virus promoter is selected from a cassava vein mosaic virus promoter, a CaMV 35S promoter, a Figwort Mosaic Virus promoter, a rice actin promoter, a phaseolin promoter, an Arabidopsis thaliana Ubiquitin 10 promoter, a maize ubiquitin promoter, an Arabidopsis thaliana Act2 promoter, an Arabidopsis thaliana Ubiquitin 11 promoter, and an Arabidopsis thaliana Ubiquitin 3 promoter.

4. (Currently Amended) A method of controlling herbicide resistance in weeds in a field, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of crop plants comprising ~~[[an]]~~the expression cassette of claim 1.

5. (Currently Amended) A method of selecting at least one transgenic plant that is resistant or tolerant to both phenoxy auxin and pyridyloxy auxin herbicides, said method comprising applying a herbicidically effective amount of an aryloxyalkanoate herbicide to a plurality of plants in a field, wherein said plurality of plants in said field comprises ~~comprising~~ at least one plant that is stably transformed with the expression cassette of claim 1 and is able to grow in said effective amount of the aryloxyalkanoate herbicide, and identifying said plant that is stably transformed with said expression cassette.

6. (Original) The method of claim 4, wherein said plurality of crop plants are dicots.

7. (Currently Amended) The method of claim 6, wherein said dicots are selected from the group consisting of soybean plants, cotton plants, canola plants, sugar ~~[[beets]]~~ beet plants, tomato plants, and sunflower plants.

8. (Currently Amended) The method of claim 4, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, and wherein pyridyloxy auxin herbicide comprises ~~[[is]]~~ one or more pyridyloxyacetate herbicides.

9. (Currently Amended) The method of claim 8, wherein said pyridyloxyacetate herbicide(s) is selected from the group consisting of triclopyr and fluroxypyr.

10. (Currently Amended) The method of claim 4, wherein said applying step ~~method~~ comprises applying said herbicide to said crop plants ~~[[field]]~~ within 14 days of planting a seed in said ~~[[crop]]~~ field, wherein said seed comprises an expression cassette comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein said polynucleotide encodes a protein that catalyzes degradation of a herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein said protein has at

least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4~~the expression cassette of claim 1.~~

11. (Currently Amended) The method of claim 4, wherein said plurality of crop plants further ~~comprise~~comprises a second ~~heterologous~~ polynucleotide encoding a second protein that confers said crop plants with resistance to at least one other herbicide, wherein said second polynucleotide is heterologous, and said method further ~~comprise~~comprising applying said at least one other herbicide to least a portion of said field.

12. (Original) The method of claim 11, wherein said herbicides are applied sequentially or concurrently.

13. (Original) The method of claim 11, wherein said herbicides are applied from a tank mix.

14. (Original) The method of claim 11, 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, pyriithiobac, pyroxasulfone, pyroxsulam,

quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepaloxymid, terbacil, thiazopyr, thidiazuron, thiencazone, thifensulfuron, thiobencarb, topramezone, tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflurosulfuron, and tritosulfuron.

15. (Original) The method of claim 4, wherein said weeds are glyphosate-resistant.

16. (Original) The method of claim 15, wherein said crop plants are glyphosate tolerant crop plants.

17. (Currently Amended) The method of claim 4, wherein said herbicide is [[a]]the phenoxy auxin herbicide.

18. (Original) The method of claim 17, wherein said phenoxy auxin is selected from the group consisting of MCPA and a 2,4-D herbicide.

19. (Currently Amended) A method of controlling volunteer plants in a field, wherein said method comprises applying to said volunteer plants at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of transgenic crop plants comprising [[an]]the expression cassette of claim 1.

20. (Currently Amended) The method of claim 19 wherein said volunteer plants are glyphosate-resistant ~~volunteer~~ and of a different species than said transgenic crop plants.

21. (Currently Amended) A polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter wherein the polynucleotide[[that]] encodes a protein having aryloxyalkanoate dioxygenase activity, and wherein the polynucleotide has 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.~~

22. (Currently Amended) A polynucleotide optimized for expression in a plant wherein said polynucleotide is operably linked to a heterologous plant promoter or a plant virus promoter and wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the polynucleotide~~a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of~~ has a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:3, and SEQ ID NO:5.

23. (Original) The polynucleotide of claim 22 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

24. (Currently Amended) An isolated polynucleotide that is operably linked to a heterologous plant promoter or a plant virus promoter and wherein the polynucleotide[[that]] encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and a pyridyloxy auxin, wherein the polynucleotide~~a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a~~ has 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.

25. (Cancelled)

26. (Currently Amended) The polynucleotide of claim [[25]]24 wherein said promoter is a cassava vein mosaic virus promoter.

REMARKS

(I) CLAIM AMENDMENTS

Claim 25 has been cancelled without prejudice. Claims 1, 3-5, 7-11, 17, 19-22, 24, and 26 have been amended. Support for the amendments can be found in the originally filed claims and throughout the specification as filed. Applicant respectfully requests entry of the amendments.

(II) INFORMATION DISCLOSURE STATEMENT

The Examiner's statements regarding Information Disclosure Statements are noted.

(III) SPECIFICATION

The Examiner objects to the specification citing an informality on page 105. The Examiner states that the table on page 105 is not numbered and requests correction. In accordance with MPEP § 714, an amendment is submitted herewith to number the table on page 105 of the specification. Specifically, please add "Table 26.b" below the sentence, "Below is a subset of the data for some of the cotton callus that has been tested." and above the table.

(IV) CLAIM OBJECTIONS

The Examiner has objected to claims 4, 10, and 19. Specifically, the Examiner objects to claim 10 stating that it is in improper form because a multiple dependent claim should refer to other claims in the alternative. Claim 10 has been amended so that it refers to only one

claim, and thus, the Examiner's objection has been rendered moot. Withdrawal of the objection is respectfully requested.

The Examiner objects to claims 4 and 19, stating that the phrase "an expression cassette" should read – the expression cassette—in referring to claim 1. Claims 4 and 19 have been amended to comply with this request. Thus, the Examiner's objections have been rendered moot. Withdrawal of the objections to claims 4 and 19 is respectfully requested.

(V) REJECTION UNDER 35 U.S.C. § 101

The Examiner has rejected claims 1, 2, and 21-24 under 35 U.S.C. § 101 arguing that the claimed invention is directed to a judicial exception (i.e. a law of nature, a natural phenomenon, or an abstract idea) without significantly more. Specifically, the Examiner argues that claims 1, 2, and 21-24 are directed to a product of nature. In response to the Examiner's rejection, claims 1, 21, 22, and 24 have been amended to recite a "heterologous plant promoter" or a "plant virus promoter." Claims 2 and 23 depend from claims 1 and 22, respectively, and, accordingly, these claims now include a reference to a heterologous plant promoter or a plant virus promoter. Applicant contends that a heterologous promoter and a plant virus promoter linked to polynucleotides that the promoters are not inked to in nature, are not naturally occurring polynucleotide constructs. Withdrawal of the rejection of claims 1, 2, and 21-24 under 35 U.S.C. § 101 is respectfully requested.

(VI) REJECTION UNDER 35 U.S.C. § 112(a) and § 112(b)

The Examiner has rejected claims 21-26 under 35 U.S.C. § 112(a), as failing to comply with the written description requirement and the Examiner rejected claims 8 and 9 under 35 U.S.C. § 112(b) for failure to distinctly claim the subject matter which the inventor regards as the invention. Applicant does not agree with the Examiner's rejections under § 112. Applicant respectfully traverses the Examiner's rejections under 35 U.S.C. § 112 (a) and (b). However, in an effort to expedite prosecution of the captioned application, in response to the rejection under 35 U.S.C. § 112 (a), Applicant has amended independent claims 21, 22, and 24 to delete the language objected to by the Examiner. Claim 23 depends from claims 22 and claim 26 depends from claim 24; and thus, these claims are similarly amended.

In response to the Examiner's rejection of claims 8 and 9 under 35 U.S.C. § 112(b) for indefiniteness, Applicant has amended claim 8 to clarify that the Markush group recited in claim 4 is included in claim 8; however, of the herbicides available to select from the Markush group (i.e., a pyridyloxy auxin herbicide and a phenoxy auxin herbicide), the pyridyloxy auxin herbicide group is further narrowed to comprise one or more pyridyloxyacetate herbicides while the pyridyloxy auxin herbicide group is not further narrowed.

Applicant believes the above-identified amendments respond to the Examiner's rejections under 35 U.S.C. § 112. Accordingly, withdrawal of the rejections under 35 U.S.C. § 112(a) and (b) is respectfully requested.

(VII) REJECTION UNDER 35 U.S.C. § 102(b)

The Examiner has rejected claims 1, 2, and 21-24 under 35 U.S.C. §102(b) as being anticipated by Schleinitz *et al.* (2004) taken with evidence from Maliga (2002). Applicant respectfully traverses the Examiner's rejection under 35 U.S.C. §102(b). Amended claims 1, 2, and 21-24 do not lack novelty under 35 U.S.C. § 102(b) in view of Schleinitz *et al.* taken with evidence from Maliga. Anticipation requires that each and every element of the claims be disclosed, either expressly or inherently, in a single prior art reference or embodied in a single prior art device or practice. See *In re Paulsen*, 30 F.3d 1475, 1478 (Fed. Cir. 1994); *Minnesota Min. & Mfg. Co. v. Johnson & Johnson Orthopaedics, Inc.*, 976 F.2d 1559, 1565 (Fed. Cir. 1992). There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of invention. See *Scripps Clinic & Res. Found. v. Genentech, Inc.*, 927 F.2d 1565, 1576 (Fed. Cir. 1991). Absence of any claim element from the reference negates anticipation. See *Kloster Speedsteel AB v. Crucible, Inc.*, 793 F.2d 1565, 1571 (Fed. Cir. 1986).

The Examiner contends that Schleinitz *et al.* teaches an SdpA gene which encodes instant SEQ ID NO:2 and would hybridize under "stringent conditions" with the full complement of instant SEQ ID NO:1. Further, the Examiner contends that Schleinitz *et al.* provides that the designation of an "expression cassette" merely denotes an intended use of a piece of DNA. Also, the Examiner contends that the bacterial promoters disclosed in Schleinitz *et al.* can be functional in a plant plastid. Hence, Schleinitz *et al.*, according to the Examiner, discloses the claim limitations. Applicant has amended independent claims 1, 21, 22, and 24. Specifically, claims 1, 21, 22, and 24 have been amended as previously described above to include a

polynucleotide sequence operably coupled to *a heterologous plant promoter or a plant virus promoter*. Schleinitz *et al.* does not describe a heterologous plant promoter or a plant virus promoter. Claims 2 and 23 depend from claims 1 and 22, respectively; and thus, incorporate the amendments. As a result of the above claim amendments, all of the elements of claims 1, 2, and 21-24 are not disclosed in Schleinitz *et al.* with evidence taken from Maliga. Accordingly, withdrawal of the rejection under 35 U.S.C. § 102(b) is respectfully requested.

(VIII) REJECTION UNDER 35 U.S.C. § 103(a)

The Examiner has rejected claims 1-7 and 11-26 under 35 U.S.C. § 103(a), asserting that the claims are obvious over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz *et al.*, Pallet *et al.*, (U.S. Patent 7,250,561 B1) and Xu *et al.*, (U.S. Patent 6,664,384). Applicant notes the office action states the Xu *et al.* U.S. Patent number is 6,665,384, but Applicant believes the Examiner meant 6,664,384 as evidenced from the 892 Form provided January 12, 2017. Applicant respectfully traverses the Examiner's rejections under 35 U.S.C. §103(a). Applicant does not agree with the Examiner's assessment of the prior art. Applicant has cancelled claim 25 without prejudice. Claims 1, 3-5, 7-11, 17, 19-22, 24, and 26 have been amended, and as a result the pending claims are not obvious over the combined teachings of Kaphammer, Schleinitz, Pallet, and Xu.

Kaphammer discloses the *tfdA* gene, which only shares about 31% identity with the *aad-12* polynucleotide sequence. Schleinitz *et al.* provides information on two genes encoding dioxygenases capable of degrading the phenoxypropionate derivatives dichlorprop and mecoprop (see page 5357, left column, first paragraph). However, the activities of the proteins

encoded by these genes were only assayed in bacteria. Thus, Schleinitz *et al.* does not teach that any of the proteins described in Schleinitz *et al.* can be used in the context of conferring herbicide tolerance to plants. Pallett *et al.* and Xu *et al.* do not cure the deficiencies of Kaphammer or Schleinitz *et al.*

The present invention relates to the use of polynucleotides, encoding an aryloxyalkanoate dioxygenase (AAD-12) enzyme, to provide tolerance in plants to more than one class of herbicide. The *aad-12* nucleic acid sequences (SEQ ID NOS: 1, 3, and 5) provide plants transformed with these polynucleotides tolerance to at least two classes of herbicides: phenoxyacetate auxin and pyridyloxyacetate auxin herbicides. No aryloxyalkanoate dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of these two different chemical classes, phenoxyacetate and pyridyloxyacetate auxin herbicides, in plants. Therefore, the polynucleotides specified in the claims for plant expression, possess properties that are not obvious over Schleinitz *et al.* and Kaphammer.

Kaphammer does nothing to make obvious the polynucleotides specified in the claims conferring tolerance to plants to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides. First, Kaphammer relates to *tfdA*, which shares only about 31% identity with AAD-12. So, the AAD-12 polynucleotide sequences, specified in the claims, are not obvious over Kaphammer. Also, Kaphammer mentions only tolerance to phenoxyacetate auxin herbicides, and not tolerance to pyridyloxyacetate auxin herbicides. Thus, Kaphammer provides no teaching that an *aad-12* polynucleotide could be used to provide a plant tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides.

Schleinitz *et al.* provides nothing further to make the claimed methods and compositions obvious. Schleinitz *et al.* provides information on two genes encoding dioxygenases capable of degrading the phenoxypropionate derivatives, dichlorprop and mecoprop. The activities of the proteins encoded by these genes were assayed in bacteria. Thus, Schleinitz *et al.* does not have a teaching that any of the proteins described in Schleinitz *et al.* can be used in the context of conferring herbicide tolerance to plants, let alone tolerance to two different chemical classes of herbicides, such as phenoxyacetate and pyridyloxyacetate auxin herbicides.

The above discussed unexpected suitability of *aad-12* polynucleotides to confer strong herbicide resistance in plants to two different chemical classes of herbicides, phenoxyacetate and pyridyloxyacetate auxin herbicides, is alone sufficient to substantiate non-obviousness of the subject matter of the present claims over the combination of Kaphammer and Schleinitz *et al.* However, in addition, *aad-12* has been shown to provide unexpectedly strong herbicide tolerance in plants.

Example 14 (entitled "Further Evidence of Surprising Results: *aad-12* versus *aad-2*") of the subject specification states (with emphasis added):

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* (vi) 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 . . . *aad-12* (vi) and *aad-2* (vi) 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 T1 Arabidopsis plants. **Surprisingly, *aad-2* (vi) and *aad-2* (v2) transformants were far less resistant to 2,4-D than the *aad-12* (vi) gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *aad-2* (vi) 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* (vi) 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.**

Thus, it is clear that *aad-12* surprisingly performed much better in plants than *aad-2* (which had little if any activity in plants). *aad-2* has a higher level of identity to the cited *tfdA* gene of Kaphammer so *aad-2* is more related at the sequence level to the cited *tfdA* gene than is *aad-12*.

Accordingly, not only does *aad-12* provide surprisingly strong herbicide tolerance to plants, but the expression of the claimed *aad-12* polynucleotides renders plants not only tolerant to phenoxyacetate auxins, such as 2,4-D, but also to pyridyloxyacetate herbicides, such as triclopyr and fluroxypyr (see page 4, paragraph [0015]).

Pallet *et al.* describes polynucleotides and methods for transforming Maize cells with genes that provide some level of resistance to dihalohydroxybenzonitriles, hydroxylphenyl pyruvate dioxygenase (HPPD), ESPSPS-inhibiting herbicides, and acetolactate synthase (ALS) inhibiting herbicide, altogether different herbicide classes than those claimed. Xu *et al.* describes designing promoters for expressing genes using pieces of a viral promoter. Thus, the Pallet *et al.* and Xu *et al.* references do nothing to provide any suggestion that the claimed *aad-12*

polynucleotides could be used in plants to provide tolerance to both phenoxyacetate and pyridyloxyacetate auxin herbicides, and do nothing to provide any suggestion that the *aad-12* polynucleotides specified in the claims provide surprisingly strong herbicide tolerance. In light of the surprising results described and claimed in the instant application, the withdrawal of the rejection of claims 1-7, 11-24, and 26 under 35 U.S.C. § 103(a) is requested.

(VIII) DOUBLE PATENTING REJECTIONS

(1) The Examiner has rejected claims 1-9 and 11-26 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-18 of issued U.S. Patent No. 8,916,752. Transmitted herewith is a terminal disclaimer document under 37 C.F.R. § 1.321 disclaiming any term beyond the expiration date of U.S. Patent No. 8,916,752, and stating the assignees' interest in the application. Thus, withdrawal of the obviousness-type double patenting rejection of claims 1-9 and 11-26 over claims 1-18 of U.S. Patent No. 8,916,752 is respectfully requested.

(2) The Examiner has rejected claims 1-9 and 11-26 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-28 of issued U.S. Patent No. 8,278,505. Transmitted herewith is a terminal disclaimer document under 37 C.F.R. § 1.321 disclaiming any term beyond the expiration date of U.S. Patent No. 8,278,505, and stating the assignees' interest in the application. Thus, withdrawal of the obviousness-type double patenting rejection of claims 1-9 and 11-26 over claims 1-28 of U.S. Patent No. 8,278,505 is respectfully requested.

(3) The Examiner has rejected claims 1-9 and 11-26 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-14 of issued U.S. Patent

No. 8,283,522. Transmitted herewith is a terminal disclaimer document under 37 C.F.R. § 1.321 disclaiming any term beyond the expiration date of U.S. Patent No. 8,283,522, and stating the assignees' interest in the application. Thus, withdrawal of the obviousness-type double patenting rejection of claims 1-9 and 11-26 over claims 1-14 of U.S. Patent No. 8,283,522 is respectfully requested.

(4) The Examiner has rejected claims 4, 6-9 and 11-18 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 7-12 of issued U.S. Patent No. 9,062,284. Applicant notes the office action states the U.S. Patent serial number is 9,062,294, but Applicant believes the Examiner meant 9,062,284 as evidenced from the 892 Form provided January 12, 2017. Transmitted herewith is a terminal disclaimer document under 37 C.F.R. § 1.321 disclaiming any term beyond the expiration date of U.S. Patent No. 9,062,284, and stating the assignees' interest in the application. Thus, withdrawal of the obviousness-type double patenting rejection of claims 4, 6-9 and 11-18 over claims 7-12 of U.S. Patent No. 9,062,284 is respectfully requested.

(5) The Examiner has provisionally rejected claims 4, 6-9 and 11-18 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 30-37 of copending U.S. Patent Application No. 15/184,601. Transmitted herewith is a terminal disclaimer document under 37 C.F.R. § 1.321 disclaiming any term beyond the expiration date of any patent that issues from U.S. Patent Application No. 15/184,601, and stating the assignees' interest in the application. Thus, withdrawal of the obviousness-type double patenting rejection of claims 4, 6-9 and 11-18 over claims 30-37 of U.S. Patent Application No. 15/184,601 is respectfully requested.

CONCLUSION

The foregoing amendments and remarks are believed to fully respond to the Examiner's rejections.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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<i>Confirmation No.:</i>	3336	}	
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SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

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

Sir:

This Statement is filed in the captioned application pursuant to 37 C.F.R. §1.56. The filing of this Statement shall not be construed to be an admission that the information cited in the Statement is, or is considered to be, material to patentability as defined in §1.56(b).

No representation is intended that a complete search has been made of the prior art or that no better art references than listed below are available. Pursuant to 37 C.F.R. § 1.98 (a)(2)(i) and 37 C.F.R. § 1.98 (a)(2)(ii) copies of foreign patent applications and non-patent-literature, or their abstracts, are provided with this paper for review by the Examiner.

Applicant hereby authorizes the Commissioner to charge any fees for a large entity to the Barnes & Thornburg Deposit Account No. 10-0435, with reference to our Matter No. 14764-245772.

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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE INFORMATION DISCLOSURE STATEMENT				ATTY. DOCKET NO. 14764-245772		APPLICATION NO. 14/491,197	
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U.S. PATENT DOCUMENTS							
*Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date if Appropriate
	AA	5,608,147	03-1997	Bryan J. Kaphammer			
	AB	7,205,561	6-1998	Pallet, et al.			
	AC	6,645,497	11-2003	Malvar et al.			
	AD	8,283,522	10-2012	Wright, Terry R.			
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	AF						
	AG						
	AH						
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FOREIGN PATENT DOCUMENTS							
		Document Number	Date	Country	Class	Subclass	Translation Yes No
	AL	WO 2005/107437	11-2005	WO			
	AM	WO 2007/053482	05-2007	WO			
	AN						
	AO						
OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)							
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	AZ	Halford et al., Weed Technology, 2001, Vol. 15, p. 737-744					
Examiner						Date Considered	
<p>*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.</p>							

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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 GENESBackground 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 (Agrilience, 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 O₂ 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.

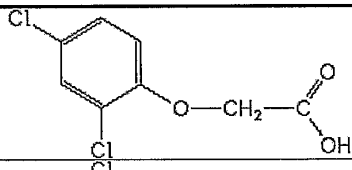
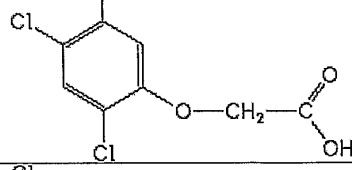
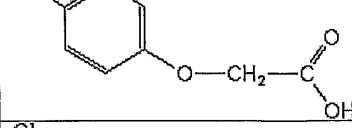
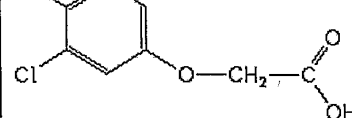
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	

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, pyrimidinylthiobenzoates, and other chemistries = *AHAS*, *CsrI*, *SurA*, *et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxyphenyl-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-I*), 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), imidazolinones (such as imazamox, imazethapyr, imazaquin), triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam),

pyrimidinylthiobenzoates (such as bispyribac and pyriothiobac), 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)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R" 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 (*CryIF*, *CryIAb*, *Cry 34/45*, other Bt. Proteins, or insecticidal proteins of a non-*Bacillus* 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 HTC. 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 ³²P-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 (T_m) 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):

$$T_m = 81.5^{\circ} \text{C} + 16.6 \text{ Log}[\text{Na}^+] + 0.41(\% \text{G+C}) - 0.61(\% \text{formamide}) - 600/\text{length of duplex in base pairs.}$$

[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 T_m-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 (T_m) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T_m for oligonucleotide probes can be determined by the following formula:

$$T_m (^{\circ}\text{C}) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(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 functionality 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 *Escherichia 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 *Bal31* 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., alfalfa 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., aryloxypropionates 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 desirable 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 known in the art, and can foreseeably 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."; Cramer 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; Cramer, 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 Cramer, 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, amiprofos-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-dinitroanilines (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 $\leq 50\%$ 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 redesign 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 MacVector™ program (Accelrys, 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
Tyrosine	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
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 Umberger, 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 #	<i>E. coli</i> Opt v2 #	Theor. Class II #	Amino Acid	Codon	Native #	<i>E. coli</i> 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		TAC	4	3	3
HIS (H)	CAC	8	11	11	TYR (Y)	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:

$$\text{Weighted \% of C1} = 1/(\%C1 + \%C2 + \%C3 + \text{etc.}) \times \%C1 \times 100$$

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 OSM 3’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 OSM 5’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; K₂HPO₄, 5 g/L; (NH₄)₂PO₄, 7.5 g/L; (NH₄)₂SO₄; MgSO₄·7H₂O, 1 g/L; KCl, 0.5 g/L; CaCl₂·2H₂O, 0.5 g/L; NaCitrate-2H₂O, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: FeCl₃·6H₂O, 5.4 g/L; MnCl₂·4H₂O, 1 g/L; ZnSO₄·7H₂O, 1.45 g/L; CuSO₄·5H₂O, 0.25 g/L; H₃BO₃, 0.1 g/L; (NH₄)₆MO₇O₂₄, 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 OD₅₇₅. 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 OD₂₈₀ 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 OD₂₈₀ 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 μ M in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μ M $\text{NH}_4(\text{FeSO}_4)_2$, 200 μ M 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 $\sim$ 500 μ M. A calibration curve performed under standard assay conditions (160 μ l final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2 μ M phenol.

[00190] Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200 μ M NH_4FeSO_4 , 200 μ M 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 μ l 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 μ l 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 $\sim$ 8,400 $\text{M}^{-1}\cdot\text{cm}^{-1}$). 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 μ M NH_4FeSO_4 , 200 μ M 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 μ l final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9 μ M 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 μ M NH_4FeSO_4 , 200 μ M 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 μ l final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1 μ M 2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be $K_m = 31 \pm 5.5 \mu\text{M}$ and $k_{\text{cat}} = 16.2 \pm 0.79 \text{ min}^{-1}$.

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

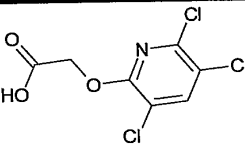
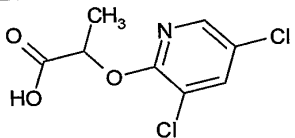
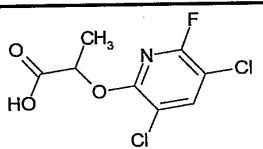
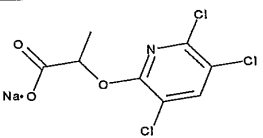
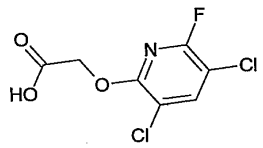
[00196] Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4 μ g purified AAD-12 (v2). (R,S)-dichlorprop (R,S-DP) was tested at 1 mM and (R)-dichlorprop, (S)-dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in **Figure 3**, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4 μ g 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 (v ₂) 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/m²sec 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 T₁ seed [*AAD-12 (v1)* gene] was allowed to dry for 7 days at room temperature. T₁ 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 T₁ 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, T₁ plants (cotyledon and 2-4-leaf 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 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 µE/m²s⁻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] T₁ 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 T₁ 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 T₁ plants with use of a track sprayer in a 187 L/ha spray volume. T₁ plants that exhibited tolerance to 2,4-D DMA were further accessed in the T₂ generation.

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

[00221] The first *Arabidopsis* transformations were conducted using *AAD-12 (v1)* (plant optimized gene). T₁ transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T₁ 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. T₁ 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* T₁ 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 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/CryIF*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The *AAD-12 (v1)* gene imparted herbicide resistance to individual T₁ *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/CryIF*-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				% 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	
Averages	<20%	20-40%	>40%	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				% Injury	
Averages	<20%	20-40%	>40%	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				% 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	
T₄ 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

[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 T₄ generation *Arabidopsis* seed (homozygous for *AAD-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 followed 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 described. 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 T₁ events were self-pollinated to produce T₂ seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T₂ siblings. Each individual T₂ 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 T₁ families (T₂ 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 T₂ individuals (T₃ seed). Twenty-five T₃ siblings from each of eight randomly-selected T₂ families were progeny tested as previously described. Approximately one-third of the T₂ 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. T₂ 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 controls (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 <i>AAD-12 (v1)</i> and transformed control <i>Arabidopsis</i> plant response to various foliar-applied auxinic herbicides.		
Pyridyloxyacetic auxins		
Herbicide Treatment	Ave % Injury 14DAT	
	Segregating T ₂ <i>AAD-12 (v1)</i> 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 <i>Arabidopsis</i> 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] T₁ *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (*AAD-12* (v1) + *EPSPS*) which encodes a putative glyphosate resistance trait. T₁ transformants were selected using *AAD-12* (v1) as the selectable marker as described in example 7.6. T₁ 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* T₄ homozygous lines (pDAB724-transformed), and *PAT* + *CryIF* 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 differencing 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. T₁ *Arabidopsis* response to a range of glyphosate rates applied postemergence (14 DAT).

AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)				% Injury	
	<20%	20-40%	>40%	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%	20-40%	>40%	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%	20-40%	>40%	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		% Injury	Ave	Std Dev
	<20%	20-40%	>40%		
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 F₁ seed was collected. Eight F₁ seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F₁ 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 F₂ seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F₂ 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-dichlorprop 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 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).

[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 novel 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 Nco1/Sac1 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 DH5 α 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 Not1 and Pvu1 to liberate the gene cassette and digest the unwanted backbone. This was ligated to Not1 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, F₁ 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 AgNO₃, 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 volume 8.1 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 was then 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 ($13 \mu\text{Em}^{-2}\text{s}^{-1}$) for one week, then higher light ($40 \mu\text{Em}^{-2}\text{s}^{-1}$) 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 T₁ 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. T₀ plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (*AHAS* gene) indicated insertion complexity ranging from 1 to >10 copies. Thirteen events contained the complete coding region for *AAD-12*; however, further analysis 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 MgCl₂ (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 T₀ Corn.

[00270] Four T₀ 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 T₀ 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 <i>AAD-12</i> .						
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

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

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 2,4-D + glyphosate (see Table 16).

Table 16. Brace Root injury of <i>AAD-12 (v1)</i> 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	<i>AAD-12 (v1)</i> pDAB4101(0)003.003	<i>AAD-12 (v1)</i> pDAB4101(0)001.001	<i>AAD-12 (v1)</i> 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)* T₁ 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 $\geq$ 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 elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F₁ seed was collected. The seed from two F₁ 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 F₁ 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 <i>AAD-12 (v1)</i> and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).				
Herbicide	Untransformed Control	2P782 (Roundup Ready Control)	<i>AAD-12 (v1)</i> pDAB4101(0) 003.R003.AF	<i>AAD-12 (v1)</i> 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) control 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 F₂ 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 normal 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 <i>AAD-12</i> events and wild-type corn following foliar applications of 2,4-D, triclopyr and fluroxypyr under field conditions.					
Treatment	Rate (g ae/ha)	%Visual Injury 14DAT			2P782 control
		AAD-12 4101(0)003.R.003.AF	AAD- 124101(0)005.001.AF		
Untreated	0	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 H₂SO₄. 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 sterily for use in the transformation protocol. Pieces of approximately one square centimeter were sterily 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 $\mu\text{E}/\text{m}^2\text{s}^{-1}$).

[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 T₀ tobacco

[00295] T₀ 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 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 20. Tobacco T0 events transformed with pDAS1580 (*AAD-12* (*v1*) + *PAT*)

#	Plant ID	Copy #	PTU PCR	Full PTU and	Full PTU and	Relative Herbicide Tolerance [@]
Tube		<i>PAT</i>	<i>AAD-12</i>	Under 2	1 copy	
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 T₁ tobacco.

[00297] Two to four T₀ 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 T₁ seed. The T₁ 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 *T₁* 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 *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.

[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.

<u>Herbicide Treatment</u>		<u>Average % Injury across locations at 14 DAT</u>			
<u>Active Ingredient</u>	<u>Rate</u>	<u>Wild type</u>	<u>pDAS1580-[1]-004.001</u>	<u>pDAS1580-[1]-020.016</u>	<u>pDAS1580-[1]-018.001</u>
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. T₁ 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. T₁ 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 <i>AAD-12</i> (<i>v1</i>) and <i>AAD-1</i> (<i>v3</i>) against elevated rates of 2,4-D.			
	KY160 control	<i>AAD-1</i> (<i>v3</i>)	<i>AAD-12</i> (<i>v1</i>)
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 F₁ seed was collected. The F₁ seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regime 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 T₂ 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 (phenoxyacetic acids + phenoxypropionic acids vs phenoxyacetic 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 stacking a second tolerance gene for the same herbicide. Such can be done using the same gene with the same or different promoters; however, as observed here, stacking and tracking two complementary traits can be facilitated by the distinguishing cross protection to phenoxypropionic acids [from *AAD-1* (*v3*)] or pyridyloxyacetic acids [*AAD-12* (*v1*)]

Table 24. Comparison of auxinic herbicide cross tolerance of *AAD-12 (v1)* (pDAS1580) and *AAD-1 (v3)* (pDAB721) T₂ plants compared to *AAD-12* x *AAD-1* F₁ cross and to wildtype.

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

Example 11 - Soybean Transformation

[00311] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgett *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 (Hinchey *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 (Hinchey *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 (Hinchey *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 produce 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 Phytigel (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 Phytigel. 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 Conviron. 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 T₀ 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 T₀ 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 trifoliolate. 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. T₀ plants will be allowed to self fertilize in the greenhouse to give rise to T₁ seed. T₁ plants (and to the extent enough T₀ 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 T₀ 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. T₁ plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T₀ and T₁ 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 T₁ 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 T₀ 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 T₁ and T₂ progeny segregation with respect to herbicide tolerance as described in previous examples.

[00327] A subset of the initial transformants were assessed in the T₀ 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 as 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. Wildtype 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. T₀ soybean response to 2,4-D leaf paint and 2,4-D spray application.

Construct (pDAB#)	Leaf flip assay 2,4-D @ (18 HAT)												
	LEAF PAINTED	Node		Spray POST over the top with 2,4-D (g ael/ha)	Stage at appl (# nodes)	ELISA (ng/mL)	Southern Copy number	PCR coding region	% injury 2 DAT	% injury 7 DAT	% injury 14 DAT		
		N-1	N-2										
4464	AAD-12	CsVMV	D-1-14	N-1	0	0	0	5246.83	2	+	X	X	0
4464	AAD-12	CsVMV	D-2-9	N-2		0	0	204.27	1	+	X	X	0
4468	AAD-12	AtUbi10	D-3-7	N-2		0	0	4.65	1	+	0	0	0
4468	AAD-12	AtUbi10	D-4-11B	N-2		0	0	1452.84	2	+	0	0	0
4468	AAD-12	AtUbi10	D-4-16	N-2		0	0	653.21	2	+	X	X	0
4480	AAD-12	AtAct2	D-9-1	N-2		0	0	248.33	3 or 4	+	X	X	0
4464	AAD-12	CsVMV	D-2-14	N-2		0	560	4917.43	2	+	0	0	0
4468	AAD-12	AtUbi10	D-3-5	N-2		0	560	365.75	1	+	0	0	0
4468	AAD-12	AtUbi10	D-3-6	N-1	0		560	714.79	3	+	0	0	0
4472	AAD-12	AtUbi3	D-5-2	N-1	0		560	0.58	1	+	5	0	0
4468	AAD-12	AtUbi10	D-3-9	N-2		0	1120	2657.26	3	+	0	0	0
4468	AAD-12	AtUbi10	D-4-17	N-2		0	1120	286.14	5	+	5	0	0
4499	PAT	CsVMV	D-2-3	N-2		1	0	2.36	5	+	X	X	0
Maverick	WT		WT-10	NT		1	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-2	NT		1	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-3	NT			0	ND	ND	ND	0	0	0
Maverick	WT		WT-4	NT			0	ND	ND	ND	0	0	0
Maverick	WT		WT-5	NT			560	ND	ND	ND	50	60	60
Maverick	WT		WT-6	NT			560	ND	ND	ND	70	90	80
Maverick	WT		WT-7	NT			560	ND	ND	ND	70	80	80
Maverick	WT		WT-10	NT			1120	ND	ND	ND	70	90	100
Maverick	WT		WT-8	NT			1120	ND	ND	ND	70	95	100
Maverick	WT		WT-9	NT			1120	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 – T₁ Progeny evaluation. T₀ plants will be allowed to self fertilize to derive T₁ 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. Surviving 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 thorough *Agrobacterium*- mediated cot-node transformation system. The T₀ 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 soybean 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 be anticipated according to methods described previously by Terakawa *et al.* (2005)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean 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% phytigel 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). Regenerated 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 $\mu\text{E}/\text{m}^2$, 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 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.

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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								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10mg/ml)				0.5 ml	0.3 ml			10 grams
Peptone								10 grams
Yeast Extract								5 grams
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 T₁ seed or outcrossed with an elite cotton line to produce F₁ 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 traits as described in experiments 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 transformation of rye, *see, e.g.*, Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.*, Hinchey *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 tuberosum*), Sweet potato (*Ipomoea batatas*), 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 sativa*), 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*, *Cynodon*, and other genera), Clover (*Trifolium*), 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), H₂O 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 T₁ 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 T₁ *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 T₁ 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 Agrilience 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 mixes of 70-1120 or 35-560 g ae/ha of triclopyr and fluroxypyr, respectively, to increase spectrum and/or increase the ability 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 Agrilience (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 HTC's 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. Triclopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimen. 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC's 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 (Agrilience, 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 canadensis*) 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 g 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC's 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 (Agrilience, 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)*, monocot 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC's 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 Phytigel (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 T₀ 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 T₁ rice

[00398] A 100-plant progeny test was conducted on five T₁ 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 T₁ Rice.

[00400] The following T₁ *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. T₁ <i>AAD-12 (v1)</i> and untransformed control response to varying levels of 2,4-D DMA.			
	Lemont Untransformed Control	pDAB4101(20)003	pDAB4101(27)002
Herbicide	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 fluorometer (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 T₀ 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 T₁ 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 excellent 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 T₁ generation rice plants response to 2,4-D and triclopyr under field conditions.*

Herbicide Treatment		% Visual Injury			
Active Ingredient	Rate	7DAT		15DAT	
		<i>AAD-12</i> event pDAB4101[20]	Wild-type Clearfield	<i>AAD-12</i> 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 <i>AAD-12 (v1)</i> 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 T₀ Canola.

[00423] Forty-five T₀ 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 T₁ (and T₂ 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 T₁ 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 T₁ Canola

[00428] For T₁ 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 T₁ 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. T₁ *AAD-12* (*v1*) and untransformed control response to varying rates postemergence 2,4-D DMA applications.

	Untransformed Control	pDAB3759(33)013.001	pDAB3759(18)009.001	pDAB3759(18)022.001	pDAB3759(18)030.001	pDAB3759(18)023.001
Herbicide	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.

<u>Herbicide Treatment</u>		<u>% Visual Injury at 14 DAT</u>		
Active Ingredient	Rate	<i>AAD-12</i> event 3759(20)018.001	<i>AAD-12</i> 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 prevalent 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 Agrilience (2005). Each alternative herbicide enabled for use in HTC 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 the 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 as 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, 100, 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 OD⁶⁶⁰ were mixed with 100 ul of *Agrobacterium* (either EHA101 or LBA4404 strains) suspension at 1.0 OD⁶⁶⁰ 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 2,4-D tested above 10 mg/L; however several fast growing colonies were found in 2, 5, and 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 cotyledonary 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, pyriothiac, pyroxasulfone, pyroxulam, quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxym, terbacil, thiazopyr, thidiazuron, thiencazone, 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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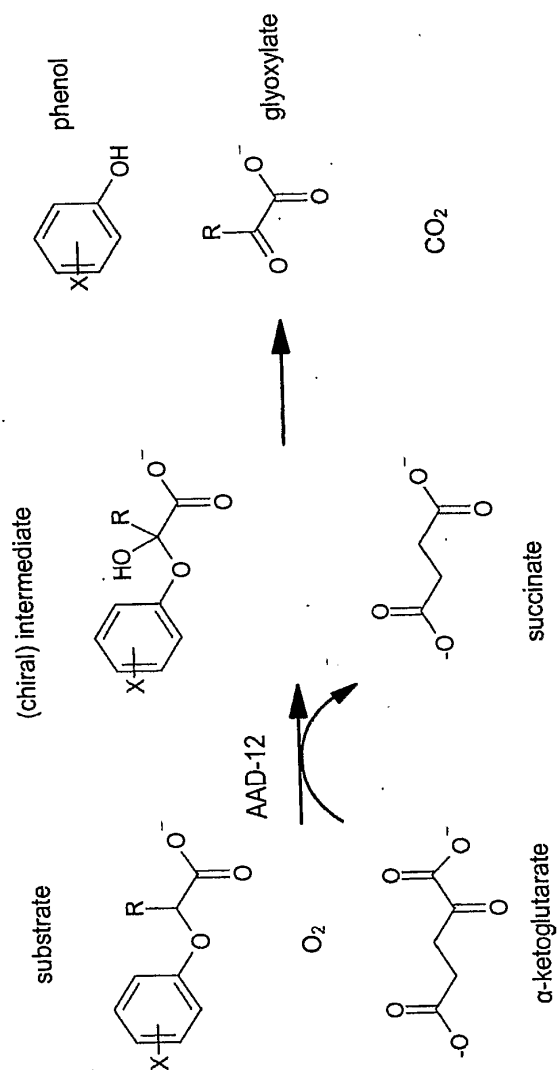


FIG. 1

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AAD-1	1	-----	MHAALSP	LSORFER	TAVQPL	TGVLG	EAELTGND
taud	1	-----	-MSERLS	HTPLPGVICA	QILSCADL	TRPLSLN	QOEQLYEA
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tfda	67	-----	-GFIKVNQ	RFRKFLA	DISNVSD	IGKAP	ADAREV
AAD-2	69	-----	RGCTVTQ	KQVRLO	-SGLAND	SVNLGDK	QPKADSR
AAD-1	75	-----	-----	PVDV	VELKST	IGCYE	VEVQMR
taud	65	-----	-----	ELHIHP	VYPHAE	GDEILV	DTHNDPP
	170	180	190	200	210	220	230
							
AAD-12	141	-----	RAYDAL	DEATRL	LVHQ	SARISLV	QS
tfda	147	-----	RAYDAL	PRDIQ	SELEG	RAEHVAL	NSRF
AAD-2	148	-----	RAYDAL	DDDETA	KELE	LVCHSL	MSVSRG
AAD-1	144	-----	YTAWETH	SPTWQ	ATTEGH	VAHSA	TFRVGS
taud	132	-----	TAAYEAL	SVFPRL	LSGL	RAEHDF	FRNSFP
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tfda	217	-----	HVEGL	PAEGR	MLAE	LEHAT	QREPV
AAD-2	218	-----	KTAS	SVPEGR	LITL	LDNEHA	TOPEV
AAD-1	224	-----	PLEG	MTA	ASKP	HLQF	TEHARF
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FIG. 2

SUBSTITUTE SHEET (RULE 26)

3/3

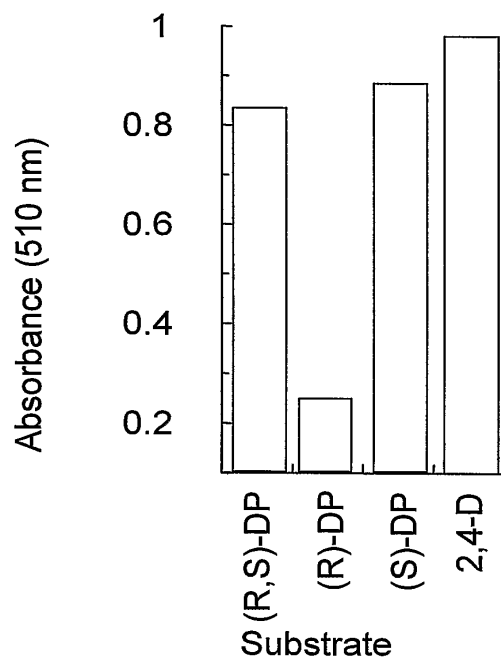


FIG. 3

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Walsh, Terence A.
Merlo, Donald J.
Jayakumar, Pon Samuel
Lin, Gaofeng

<120> Novel Herbicide Resistance Genes

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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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Department of Environmental Microbiology, UFZ Centre for Environmental Research Leipzig-Halle, Leipzig, Germany,¹ and Institut National de la Recherche Agronomique² and Laboratoire de Génétique des Microorganismes Pathogène, Institut Pasteur,³ Paris, France

Received 11 March 2004/Accepted 20 May 2004

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 Tn501-like transposon. Furthermore, two copies of a sequence similar to IS91-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 IS91-like elements. Two other dichlorprop-degrading bacterial strains, *Rhodospirillum rubrum* 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 (α KG)-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) α KG-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* 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 α KG-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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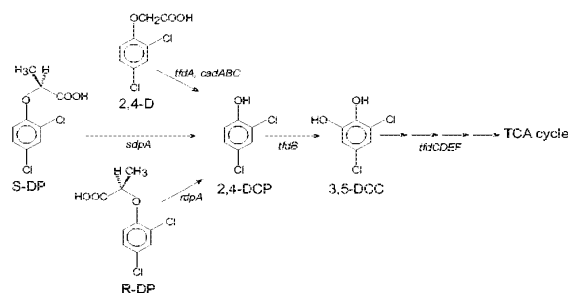


FIG. 1. Modified *ortho*-pathway for the degradation of chlorinated phenoxyalkanoate herbicides. 2,4-D, 2,4-dichlorophenoxyacetate; R-DP and S-DP, *R*- and *S*-enantiomers of 2(2,4-dichlorophenoxy)propionate, respectively; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; TCA, tricarboxylate.

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 *Rhodospirillum rubrum* 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- μ m-pore-size Minisart cellulose acetate filter (Sartorius) and added at a concentration of 100 μ g/ml.

Mercury resistance was examined on LB agar plates containing 0.1 mM HgCl₂ 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 miniTn5 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 (OD₆₀₀) of 1.0. Equal volumes of the donor and the recipient were mixed, and 100 μ l 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 μ l of the 10⁻³ to the 10⁻⁵ dilutions was plated on selective MM agar plates containing 100 μ g 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 TautD, 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 μ l 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.fg* 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 *Rhodospirillum rubrum* sp. strain P230 were amplified with primers *rdp.f* (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 Tn5 transposon insertion sites were amplified by ST-PCR (6). To each sample either Tn5.outF (5'-GGC AGA TCT GAT CAA GAG-3' [27]) annealing to the I end of the transposon or Tn5.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 *IS91*-like element was partially amplified by using the primers *IS.f* (5'-ATG TCA GCC ATT 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

TABLE 1. Degenerate primers designed for the amplification of *rdpA* and *sdpA*

Peptide or primer	Sequence ^a																Position ^b
RDP_internal sequence 2 rdp_r	W	E	T	L	S	P	T	M	Q	A	T	I	E	G	L	N	162–177
	3'-AC GTY CGN TGN TAI CTY CC-5'																
RDP_internal sequence 3 rdp_rA ^c rdp_rG ^c	N	E	I	L	D	A	F	H	T	Y	Q	V	I	Y	F	P	43–58
	5'-GCI TTY CAY ACN TAY CAA GT-3'																
	5'-GCI TTY CAY ACN TAY CAG GT-3'																
SDP_N terminus sdp_rC ^c sdp_rT ^c	M	Q	T	T	L	Q	I	T	P	T	G	A	T	L	G	A	1–16
	5'-ATG CAR ACI ACI CTG CAR ATC AC-3'																
	5'-ATG CAR ACI ACI CTG CAR AIT AC-3'																
SDP_internal sequence 1 sdp_rA ^c sdp_rG ^c	L	G	H	V	Q	Q	A	G	S	A	Y	I					193–204
	3'-GTA CAN GTY GTY CGN CC-5'																
	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).

tfpD gene fragments was performed as described in reference 35. Fragments of *tfpD* and *tfpE* 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 (Pierce Biotechnology). 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 H₂O, 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 Centr-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 (*tfpD*, *tfpE*, and *tfpF* probes), 55°C (*rdpA* probe), 60°C (probes against *tfpB*, the *mer* operon, and the IS91 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-

TABLE 2. Sequence identities of the dichlorprop-cleaving enzymes RdpA and SdpA to other group II α KG-dependent dioxygenases

α -KG dioxygenase ^a	Strain, accession no.	% Identities ^b to	
		RdpA	SdpA
TtdA	<i>Ralstonia eutropha</i> , A27082	32/279	37/244
TauD	<i>Pseudomonas aeruginosa</i> , NP_252624	35/279	27/258
AtsK	<i>Pseudomonas putida</i> , AAD31784	31/296	31/296
YSD	<i>Saccharomyces cerevisiae</i> , Q12358	29/279	27/304
PvcB	<i>Pseudomonas aeruginosa</i> , AAC21672	20/298	21/277
SyrI	<i>Pseudomonas syringae</i> , AAB63253	17/323	18/308
CarC	<i>Pectobacterium carotovorum</i> , AAD38231	16/285	17/275
γ -BBH	<i>Pseudomonas</i> sp. strain AK-1, P80193	16/309	17/291
CS2	<i>Streptomyces clavuligerus</i> , AAA26723	16/208	17/275

^a TtdA, 2,4-D/ α KG dioxygenase; TauD, taurine/ α KG dioxygenase; AtsK, putative α KG-dependent alkylsulfatase; YSD, sulfonate/ α KG dioxygenase; PvcB, pyoverdine biosynthesis enzyme; SyrI, syringomycin biosynthesis enzyme I; CarC, carbapenem biosynthesis enzyme C; γ -BBH, γ -butyrobetaine hydroxylase; CS2, clavaminic synthase 2.

^b That is, the percent sequence identity over the total number of amino acids examined.

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 α KG-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 TtdA 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-Tn5 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 Tn501 subgroup of the Tn3 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

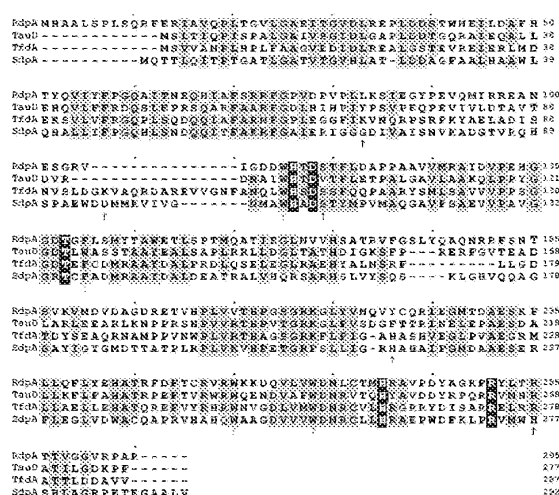


FIG. 2. Sequence alignment of RdpA and SdpA with other group II α KG-dependent dioxygenases. TtdA, *R. eutropha* 2,4-D/ α KG dioxygenase (accession no. A27082); TauD, *P. aeruginosa* taurine/ α KG dioxygenase (accession no. NP_252624). Conserved Fe(II)- and α KG-binding residues are shaded in black; other conserved regions are shaded in gray. Black arrows indicate 2,4-D binding residues in TtdA; gray arrows indicate residues undergoing posttranslational hydroxylation in TauD or TtdA. Amino acid positions numbers are shown to the right of each line. The alignment was created with CLUSTAL W.

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 Tn501 transposon showed 81% nucleotide sequence identity to a putative Tn3 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.

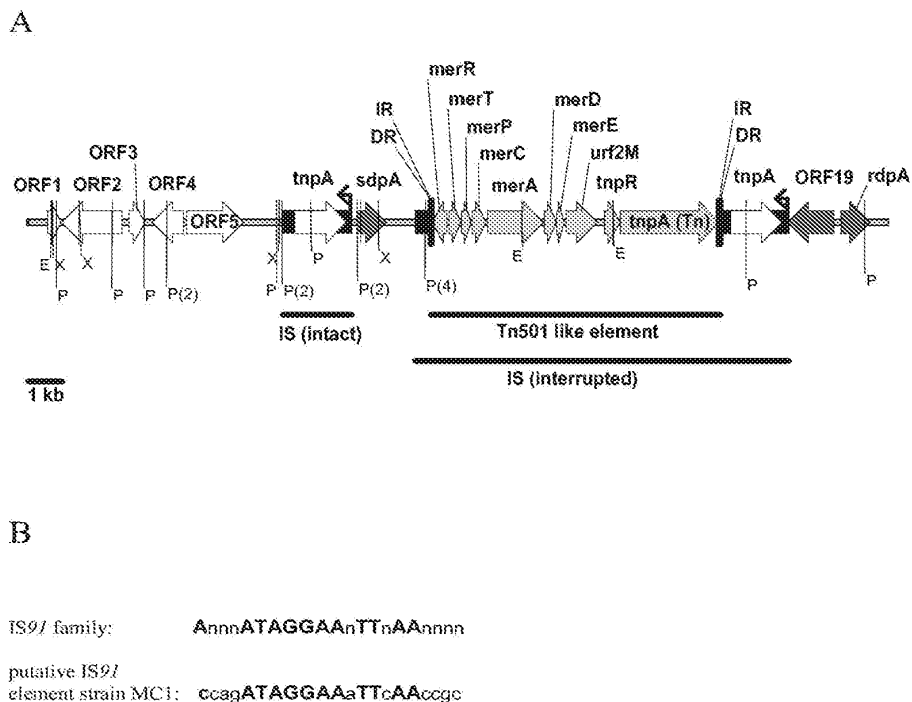


FIG. 3. Organization of *rdpA* and *sdpA* within the genome of strain MC1. (A) Genetic map of the genomic fragment containing *rdpA* and *sdpA*. For ORFs showing a high degree of similarity to other genes (>80% identity of the deduced amino acid sequence) the respective names are given. The gene designations 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 Tn501-like transposon, DR, 5-bp direct repeat. Two putative IS91-like elements are shown as black boxes; the positions of the *oriIS* are indicated as arrows above the sequence. The right IS91-like element is interrupted by a Tn501-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 IS91 family *oriIS* consensus sequence. Conserved nucleotides are shown in bold uppercase letters.

Furthermore, two copies of a putative IS element resembling the IS91/IS801 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 *oriIS*, the origin of replication, and a *terIS*, 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 Tn501-like element (Fig. 3A). The elements were 2,037 bp (intact copy) and 2,084 bp (interrupted copy) and contained an IS801-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 *oriIS* of this group of insertion elements (49) in all but the first nucleotide (Fig. 3B). Neither the termination sequence *terIS* nor the tetramers GTTC/CTTG for which IS91 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

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/matrix ^c	Accession no.
1	574-966	d	Carboxymuonolactone decarboxylase family protein	<i>Stenotrophomonas maltophilia</i> MR-1	65 (81/124)	130/128	AAN53170.1
2	1006-2799	c	Glutathione-regulated potassium-proton antiporter	<i>Pseudomonas putida</i> KT2440	69 (385/556)	597/581	AF016523.1
3	3018-3494	d	Putative bacterioferritin	<i>Delftia acidovorans</i>	70 (112/158)	158/158	CAC83620.1
4	3701-4642	c	Hypothetical protein, similar to LysR-type regulators	<i>Ralstonia metallidurans</i>	51 (161/314)	313/360	ZP_00022545.1
5	4745-6438	d	Putative dihydroxy acid dehydratase	<i>Ralstonia solanacearum</i>	76 (420/555)	564/557	CAD161119.1
6	7981-9528	d	Putative transposase, similar to IS801 transposase	<i>Pseudomonas</i> sp. strain ADP	99 (512/515)	515/515	AAK50272.1
7	9836-10714	d	2,4-D dioxygenase (TfdA)	<i>Ralstonia eutropha</i> JMP134	37 (92/249)	292/287	A27082
8	12107-12541	c	Mercury resistance regulatory protein (MerR)	<i>Enterobacter aerogenes</i>	97 (140/144)	144/144	CAN83896.1
9	12613-12963	d	Mercury uptake protein (MerF)	<i>Alcaligenes</i> sp.	96 (112/116)	116/116	CAA70196.1
10	12977-13251	d	Mercury uptake protein (MerP)	<i>Alcaligenes</i> sp.	94 (86/91)	91/91	CAA70197.1
11	13279-13701	d	Mercury uptake protein (MerC)	<i>Nitrosomonas europaea</i> ATCC 19718	86 (121/140)	140/140	CAD84751.1
12	13741-15426	d	Mercury ion reductase (MerA)	<i>Klebsiella pneumoniae</i>	88 (494/561)	561/561	AAM44226.1
13	15444-15809	d	Mercury resistance secondary regulatory protein (MerD)	<i>Pseudomonas</i> sp. strain ADP	95 (115/121)	121/121	AAK50284.1
14	15806-16042	d	Putative protein (MerE)	<i>Pseudomonas</i> sp. strain ADP	91 (71/78)	78/78	AAK50283.1
15	16093-17028	d	Putative protein, similar to Urf2M	<i>Ralstonia metallidurans</i>	88 (274/310)	311/329	CAD97548.1
16	17245-17718	d	Resolvase (TnpR)	<i>Nitrosomonas europaea</i> ATCC 19718	98 (154/157)	158/186	CAD84747.1
17	17721-20690	d	Tn3 family transposase (TnpA)	<i>Nitrosomonas europaea</i> ATCC 19718	95 (945/989)	989/989	CAD84746.1
18	21024-22571	d	Putative transposase, similar to IS801 transposase	<i>Pseudomonas</i> sp. strain ADP	99 (511/515)	515/515	AAK50272.1
19	22752-24137	c	Putative aromatic compound MFS transporter	<i>Pseudomonas putida</i> KT2440	73 (318/433)	461/450	AAN69258.1
20	24330-25217	d	Taurine dioxygenase (TadD)	<i>Pseudomonas aeruginosa</i> PA01	55 (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 IS91-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 *Rhodococcus* 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 α KG-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 α KG-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)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R/K, which is conserved throughout group II α KG-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 α KG-binding residues Thr141 and Arg274 (Fig. 2). All of these residues were conserved in RdpA and SdpA, representing the putative Fe(II) and α KG-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

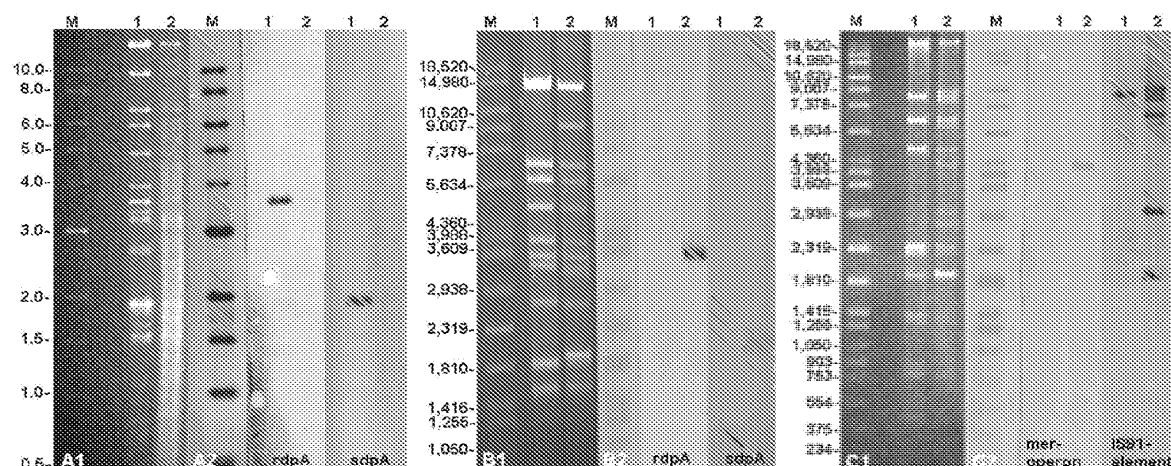


FIG. 4. Localization of *rdpA* and *sdpA* within the genome of *D. acidovorans* MC1 and comparison of plasmid pMC1 with pMC1-DP⁻, the plasmid of mutant MC1-DP⁻. (A) Localization of *rdpA* and *sdpA* within the genome of strain MC1. Plasmid DNA (lane 1) and genomic DNA (lane 2) was digested with PstI, 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 IS91 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 α KG dioxygenases a high degree of structural conservation was observed between clavamate 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/TfdA α 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 α KG dioxygenase family.

Transposon mutagenesis inactivation of *rdpA* indicated that it was a single-copy gene and gave a further proof of its func-

tionality 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 *Rhodospirillum rubrum* 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

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 IS91-like element was found to be considerably reduced. This suggests that recombination between IS91 elements could lead to the deletion of one or both dioxygenase genes and the Tn501-like transposon. Although IS91-like elements do not cause deletion of regions adjacent to them, a deletion of a genomic fragment flanked by IS91-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 led to the acquisition and causing instability of the degradative trait.

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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 basic as well as basic agricultural practice. Weed control, as practiced for millennia is basic 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.

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 oil-seed 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 *psbA* 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 *psbA* gene products were crystallized and analyzed, leading to new insights into 'drug' (ligand) binding and design, as well as a No-

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 & Morishima, 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

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 abundance 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

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 chauvinism 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 en-

gineer 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

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 conferred resistance to thiazopyr via degradation (Feng et al., 1998). The expression of plant and animal P₄₅₀ transgenes conferred 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

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. napus*) (Souza-Machado, 1982). This *psbA* 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 AC-Case 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 (Padgett 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 five-fold in chloroplasts are resistant to a discriminatory dose of 20 μ M acifluorfen, which severely inhibited the wild type (Lermontova & Grimm, 2000). Unfortunately, in this latter case, there are no quantitative data on the magnitude of resistance.

The nail in the coffin of invincibility came with a recent identification of glyphosate-resistant *Eleusine indica* in Malaysia (Lee & Ngim, 2000). This

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 transgenic resistance genes (Padgett 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 are 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 & Murrant, 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

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

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 unpredictable 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

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-familially 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

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 Kariyeva 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

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 & Rottevel, 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 in-

heritance 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 inheritance, 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 con-specific 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-

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;

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.

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 unforeseen and unforeseeable 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-

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 co-suppression 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 GA₁ and GA₄ 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

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' (Hermes & 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-

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-

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 dele-

terious 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

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

is the task of formulators 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 *Orobanch* 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 Confucius: '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-

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 O₂ 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 H₂O and D₂O 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 O₂-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 clavaminase 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.

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 proclavamine 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 O₂, the order of which is enzyme dependent (4–7). The exact

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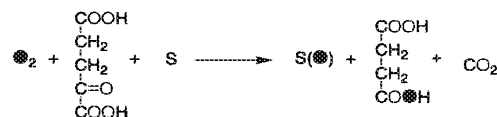
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¹ Abbreviations: CS2, clavaminase synthase 2; 2,4-D, (2,4-dichlorophenoxy)acetate; DAOCS, deacetoxycephalosporin C synthase; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; ESEEM, electron spin-echo envelope modulation; 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.

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 clavaminic 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

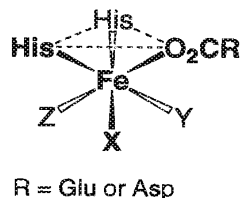
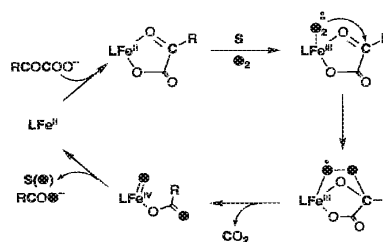
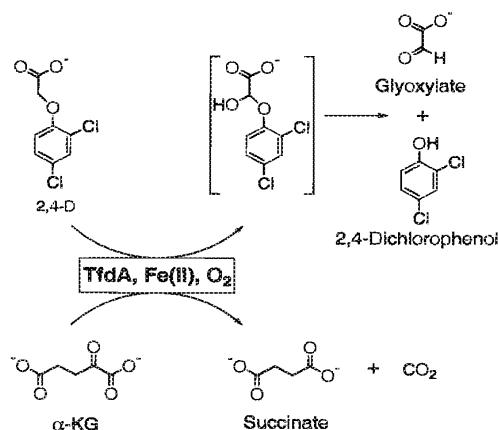


FIGURE 1: The 2-His-1-carboxylate facial triad utilized by many O_2 -activating mononuclear non-heme iron enzymes.

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_(S1)-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

other data recently obtained on this class of enzymes (8, 9, 18, 23, 24, 31, 32).

EXPERIMENTAL SECTION

Chemicals. Ferrous ammonium sulfate [$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$] and Mops were purchased from Sigma (St. Louis, MO), while 2,4-D, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 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* DH5 α 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 $30\,600\text{ M}^{-1}\text{ cm}^{-1}$ per subunit (or $1.0\text{ AU}_{280} \approx 0.96\text{ mg/mL}$) 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 $^{63}\text{CuCl}_2$ 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 $^{63}\text{CuCl}_2$ stock concentration of 12 mM. Dual samples for electron spin–echo envelope modulation (ESEEM) spectroscopy were prepared in $\text{H}_2\text{O}/\text{D}_2\text{O}$ 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 $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ 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 brilliant yellow color characteristic of iron–nitrosyl complexes. Excess NO was removed by vacuum. To ensure that adventitious 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

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 5000 G 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 μ s 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 $\times$ 1.0 cm) quartz cuvette (Wilma 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 $^\circ$ 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 μ M) 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

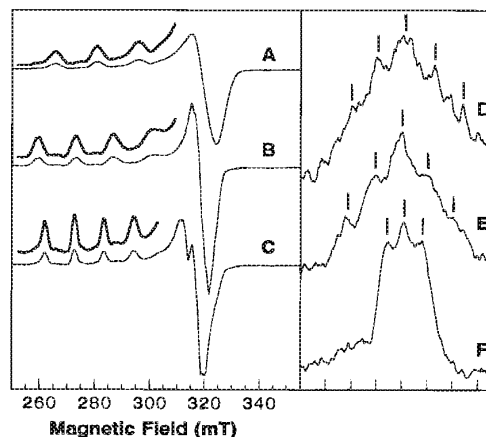


FIGURE 2: EPR spectra of Cu—TfdA complexes. (A) Cu—TfdA ($g_1 = 2.07$, $g_2 = 2.29$, $A_{||} = 16.1$ mK, 4.2 mT fwhm at 281 mT); (B) Cu—TfdA·(α -KG) ($g_1 = 2.07$, $g_2 = 2.36$, $A_{||} = 14.9$ mK, 4.2 mT fwhm at 273 mT); (C) Cu—TfdA·(α -KG)·(2,4-D) ($g_1 = 2.07$, $g_2 = 2.11$, $g_3 = 2.37$, $A_z = 12.0$ mK, 2.2 mT fwhm at 273 mT). Insets show g_1 with the intensity magnified 5 times. Spectra D–F display superhyperfine splitting of the g_1 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).

μ M). 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.

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 ($g_1 = 2.07$; $g_2 = 2.29$; $A_{||} = 16.1$ 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 $g_1 = 2.07$, $g_2 = 2.36$, and $A_{||} = 14.9$ 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 $g_1 = 2.07$, $g_2 = 2.11$, $g_3 = 2.37$, and $A_z = 12.0$ mK (Figure 2C). A comparison of the three spectra shows a progressive movement to lower field for g_2 and a decrease in the $A_{||}$ 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, $A_{||} = (g\text{-value}) \times (\text{splitting in gauss}) \times (\beta_e)$.

increase in $g_{\parallel}$ is usually indicative of a shift to a more oxyanion-rich environment (44), while small $A_{\parallel}$ values suggest a significant distortion from planarity (45, 46). For example, the $g_{\parallel}$ and $A_{\parallel}$ values of the ternary Cu-TfdA-(α -KG)·(2,4-D) complex are comparable to those of Cu-substituted ribulose-1,5-bisphosphate carboxylase/oxygenase complexed to the transition-state analogue 2-carboxyarabinitol bisphosphate [$g_{\parallel} = 2.41$, $A_{\parallel} = 11.59$ mK (47)] and Cu(II)-substituted enolase complexed to 2-phosphoglycerate [$g_{\parallel} = 2.40$, $A_{\parallel} = 12.0$ 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 ($K_m = 3.2$ μ M), 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

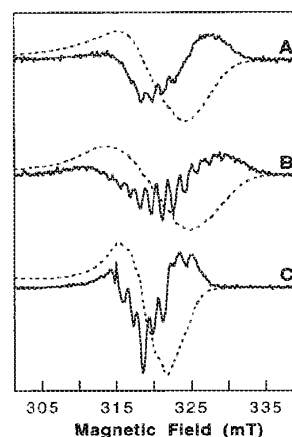


FIGURE 3: Superhyperfine splitting of the $g_{\perp}$ region in Cu-TfdA EPR spectra due to ^{14}N -containing ($I = 1$) ligands. (A) Cu-TfdA; (B) Cu-TfdA in the presence of 15 equivalents of imidazole; (C) Cu-TfdA·(α -KG). The dashed lines are the expanded first derivative $g_{\perp}$ signals, while 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 $A_{\perp}^{\text{Cu}}$ 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 $g_{\parallel}$ 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 $A_{\parallel}$ values. To increase the likelihood of observing the superhyperfine splitting, isotopically pure Cu(II) was utilized. The farthest downfield $g_{\parallel}$ 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

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 D₂O. 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 D₂O-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 D₂O 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 $\approx$ 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 ²H-ESEEM shown in Figure 4 originates from D₂O molecules bound to the metal, ambient D₂O 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 ²H fundamental and sum combination frequency components. Figure 5 shows two-pulse ESEEM simulations for D₂O 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

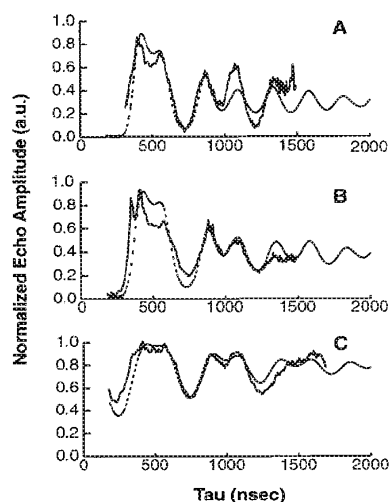


FIGURE 4: The solid lines are deuterium ESEEM data collected for (A) Cu–TfdA, (B) Cu–TfdA·(α -KG), and (C) Cu–TfdA·(α -KG)·(2,4-D). These data were obtained by dividing the two-pulse ESEEM data collected for each sample exchanged against D₂O-based buffer with the identical sample in H₂O-based buffer. 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 D₂O molecules, (B) one equatorial and one axial bound D₂O, and (C) one axially bound D₂O. Simulations used the Hamiltonian parameters for equatorial and axial bound D₂O molecules given in the captions for Figure 5, respectively.

for a D₂O 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)(H₂O)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 D₂O (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) D₂O molecules are provided. For equatorially bound D₂O, 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

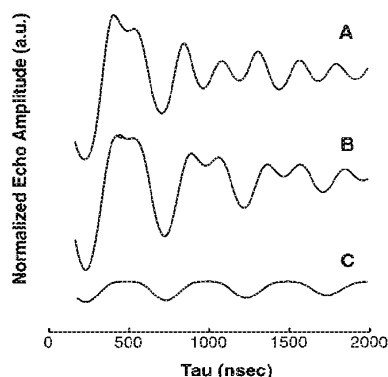


FIGURE 5: ^2H -ESEEM simulations for (A) a D_2O molecule bound equatorially to $\text{Cu}(\text{II})$, (B) an axially bound D_2O , and (C) a D_2O molecule coupled through space to $\text{Cu}(\text{II})$ so that the deuterium-copper distance was 4 Å. Hamiltonian parameters for the ESEEM simulations were for equatorially bound D_2O : $A_{\text{ex}} = -1.4$ MHz, $A_{\text{yy}} = -0.6$ MHz, $A_{\text{zz}} = 1.4$ MHz, nuclear g -value = 0.857 44, magnetic field = 0.305 T, starting $\tau = 180$ ns, $e^2qQ = 0.2$ MHz, η q asymmetry parameter = 0.5 and the η q and hyperfine tensors were taken as coaxial. Parameters were the same for the axially bound D_2O except for the hyperfine splitting, $A_{\text{ex}} = -0.5$ MHz, $A_{\text{yy}} = -0.5$ MHz, and $A_{\text{zz}} = 1.1$ MHz. For ambient D_2O (trace C), $A_{\text{ex}} = -0.2$ MHz, $A_{\text{yy}} = -0.2$ MHz, and $A_{\text{zz}} = 0.4$ MHz.

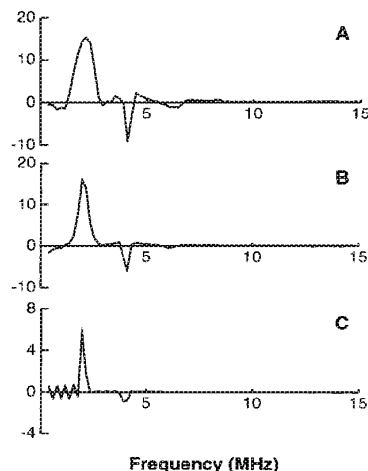


FIGURE 6: Fourier transforms of the ESEEM simulations shown in Figure 5. Trace A is for an equatorially bound D_2O , trace B an axially bound D_2O , and trace C ambient D_2O .

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 $\text{Cu}(\text{II})$ 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

Table 1: Ratios of ^2H -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

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 ^2H 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.

The 2/4 MHz amplitude ratios measured from Fourier analysis of the three TfdA ^2H -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 ^2H second harmonic is observed with these 2/4 MHz peak ratios being 1.7 and 1.8, respectively. These results favor a model for $\text{Cu}(\text{II})$ 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 $\text{Cu}(\text{II})$ site with a single, axially bound water molecule. To test this idea, ^2H -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

there is good agreement between the measured ^2H -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 $\text{Cu(II)(H}_2\text{O)}_6$ in Tutton salts (56).

There are several features of the ^2H -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 ^2H 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 ^2H -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 ^2H -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 ^2H -ESEEM pattern of the water displaced by $\alpha\text{-KG}$ binding. In a similar fashion, division of Figure 4B by Figure 4C should reveal the ^2H -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 $\alpha\text{-KG}$ 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 ^2H -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 $\text{Cu-TfdA}\cdot(\alpha\text{-KG})$ complex are more subtle, and a good ratio was obtained by dividing the ^2H -ESEEM data of Figure 4, trace B, with that of trace C. The result provides the ^2H -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 ^2H -ESEEM for a D_2O molecule bound equatorially to Cu(II) .

Binding of $\alpha\text{-KG}$ and 2,4-D to Fe(II) -Reconstituted TfdA. Binding of $\alpha\text{-KG}$ to the metal center was assessed directly via optical spectroscopy. In the absence of $\alpha\text{-KG}$, the optical spectrum of Fe-TfdA does not exhibit any absorption features in the visible region, while the UV region is dominated by protein $\pi\text{-}\pi^*$ transitions. Lack of a visible chromophore is typical of high-spin Fe(II) species. Upon the anaerobic addition of $\alpha\text{-KG}$, however, the enzyme solution

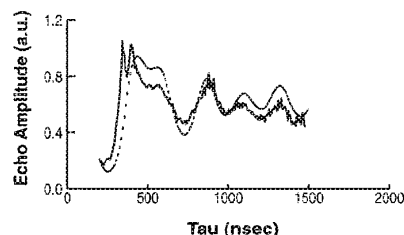


FIGURE 7: The solid line is a ^2H -ESEEM trace obtained by dividing the experimental ratios for Figure 4B by that of Figure 4C. The dashed curve is a simulation for a single D_2O molecule bound equatorially to Cu(II) and was obtained by truncating the trace of Figure 5A at 1.5 μs .

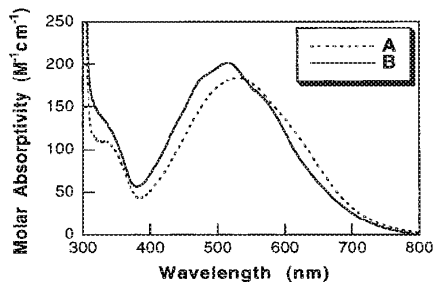


FIGURE 8: Optical absorption spectra of (A) $\text{Fe-TfdA}\cdot(\alpha\text{-KG})$ ($\lambda_{\text{max}} = 530\text{ nm}$) and (B) $\text{Fe-TfdA}\cdot(\alpha\text{-KG})\cdot(2,4\text{-D})$ ($\lambda_{\text{max}} = 515\text{ nm}$ with shoulders at approximately 475 and 560 nm). The spectrum of the 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 $\text{Fe(II)}\cdot(\alpha\text{-keto acid})$ complexes (20–22, 25) where the energy of this spectral feature is easily tuned by varying the substituent on the $\alpha\text{-keto acid}$. 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 $\alpha\text{-keto acid}$. Significantly, this feature is only observed when the $\alpha\text{-keto acid}$ chelates via the carboxylate oxygen and the $\alpha\text{-keto oxygen}$; when the $\alpha\text{-keto acid}$ binds in a monodentate fashion, the model iron complexes remain colorless (21, 22). Thus, this broad absorption feature is diagnostic of an $\alpha\text{-keto acid}$ chelated to an Fe(II) metal center, indicating that $\alpha\text{-KG}$ chelates to the iron in TfdA.

The MLCT transition observed in the presence of $\alpha\text{-KG}$ 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 ($\lambda_{\text{max}} = 515\text{ nm}$ with shoulders at approximately 475 and 560 nm). These results indicate that $\alpha\text{-KG}$ remains chelated to the Fe(II) center in the presence of substrate [i.e., in the $\text{Fe-TfdA}\cdot(\alpha\text{-KG})\cdot(2,4\text{-D})$ 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

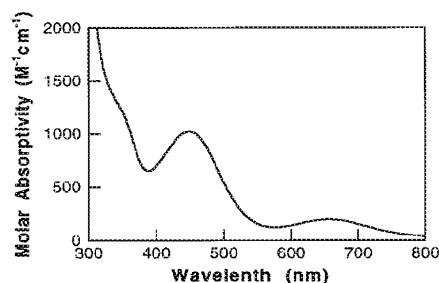


FIGURE 9: Optical absorption spectrum of Fe-TfdA-(α -KG)-(2,4-D)-(NO) ($\lambda_{\text{max}} = 449$ and 657 nm).

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 $\{\text{FeNO}\}^7$ 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 $\text{NO}^- \rightarrow \text{Fe}^{3+}$ 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 $\{\text{Fe-NO}\}^7$ 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)-(O₂) 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

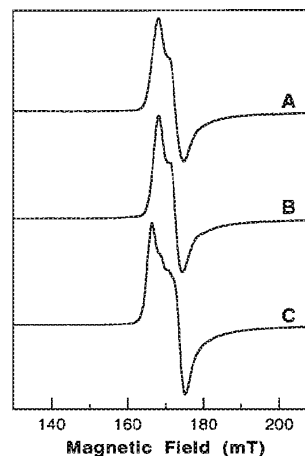


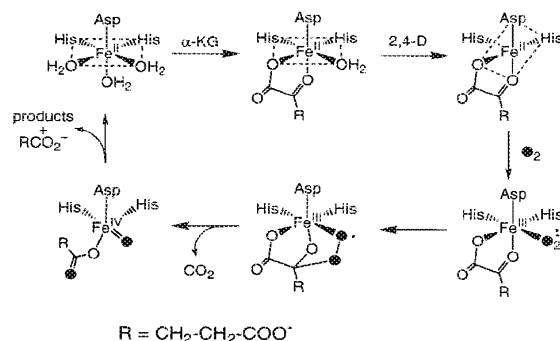
FIGURE 10: EPR spectra of Fe-TfdA-nitrosyl complexes. Only the expanded $g = 4$ region is displayed since the $g = 2$ region is relatively uninformative and there was frequent overlap with the signal of free NO. (A) Fe-TfdA-(NO) ($g_x = 4.09$, $g_y = 3.98$, $g_z = 2.00$, g_x to g_y peak-to-peak width 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).

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.

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,

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 metalcenters.

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].

sharpening of the $g_{\parallel}$ 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 oxoanionic 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 O₂. Evidence that the addition of substrate to the binary complex primes the iron to bind and activate O₂ can be derived from studies with the O₂ 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, O₂. An energetic coupling of NO/O₂ and substrate binding has been observed previously in other non-heme iron enzymes that activate O₂, including three different extradiol dioxygenases (59, 60) and IPNS (61). The increased affinity for NO/O₂ 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 O₂ 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 O₂ 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 O₂. 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 O₂ 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 O₂ (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 CO₂ 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 O₂ at the

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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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 sequence 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 polyadenylation 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

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 polyadenylation 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 polyadenylation 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 polyadenylation 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 ³²P 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-pyruvylshikimate-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 polyadenylation 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 polyadenylation 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. 2A). 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. 2A). 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 polyadenylation signal sequences.

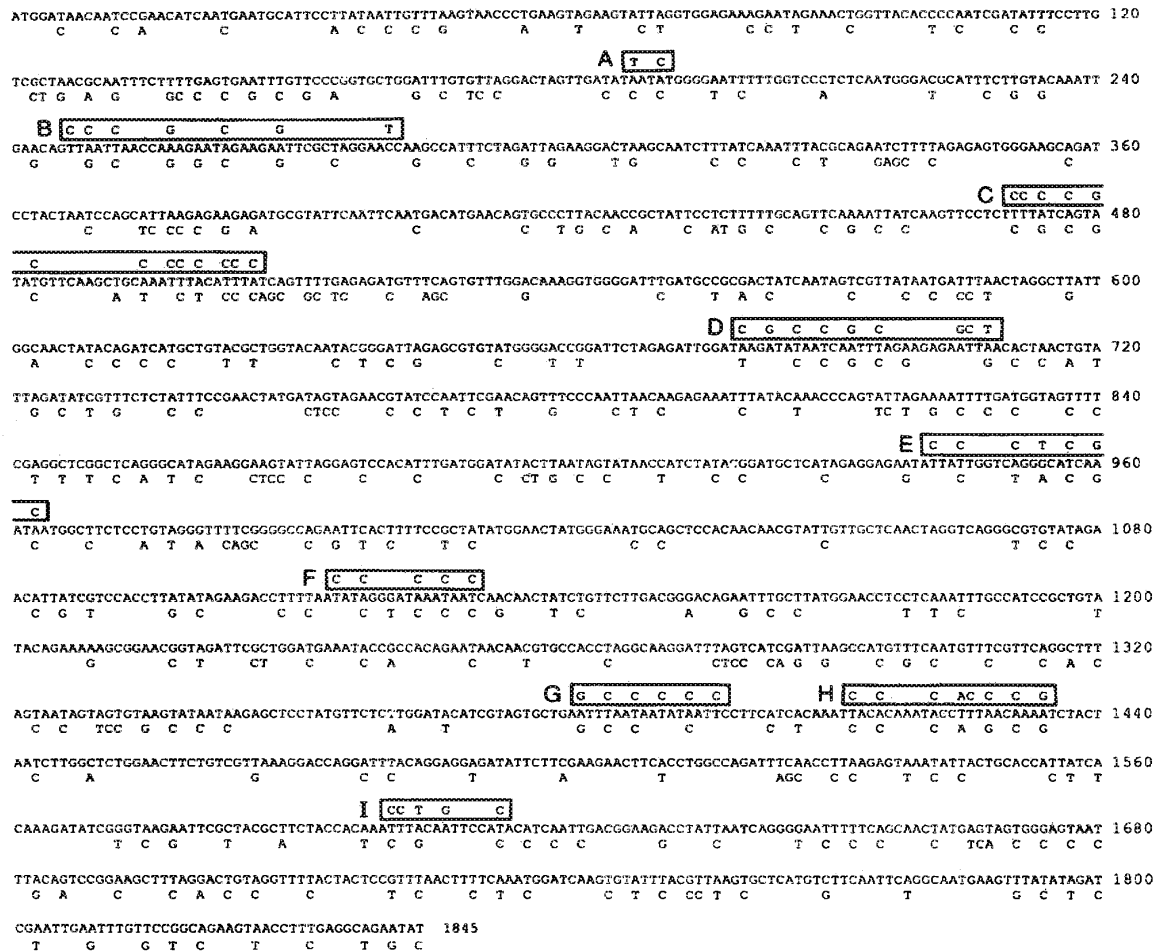


FIG. 1. DNA sequence of the *cryIA(b)* gene on the numbered line with the modifications found in the PM gene on the line above and the FM gene on the line below. The differences between the WT and PM genes are within the labeled boxed areas (A-I).

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 μ g of total protein. Among the most highly expressing transgenic tomato plants for each insect control protein 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 <i>cryIA(b)</i>	3/54 (6%)	53/204 (26%)
PM <i>cryIA(b)</i>	25/42 (60%)	40/63 (63%)
FM <i>cryIA(b)</i>	12/22 (55%)	32/55 (58%)
FM <i>cryIA(c)</i>	—	52/97 (54%)
FM <i>cryIA(c)*</i>	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.

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.

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. 2B) 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 μ g of total protein (>0.1%). Fig. 2B shows a high percentage of tobacco plants with the FM *cryIAb* 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-

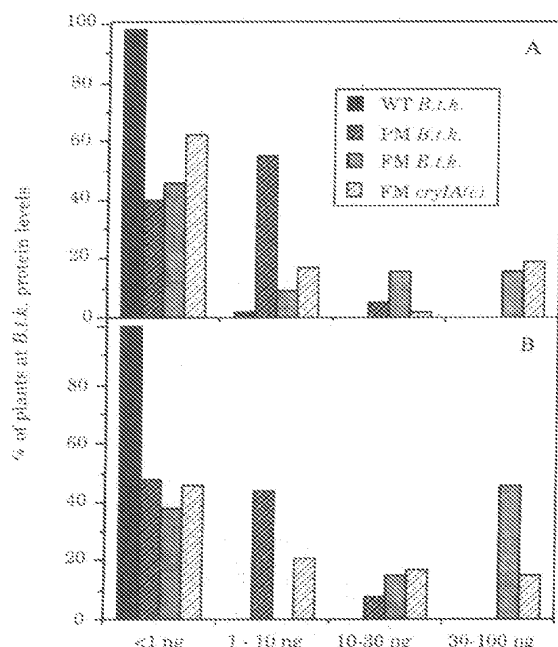


FIG. 2. Distribution of tomato (A) and tobacco (B) plants that exhibited insect control. The numbers of tomato plants that exhibited insect control used to calculate the percentage of *B.t.k.* protein levels are as follows: WT gene, 53; PM gene, 63; FM gene, 55; FM *cryIA(c)*, 97. The numbers of tobacco 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 μ g 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 μ g 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 μ g 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.

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 polyadenylation 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 polyadenylation 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 polyadenylation signal led to processing without polyadenylation 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 $\approx$ 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 $\approx$ 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 response³. 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 enzymes³⁻⁵. The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism³.

Recently, several different gene transfer approaches have been employed to improve the stress tolerance of plants⁶. The transferred genes included those encoding enzymes required for the biosynthesis of various osmoprotectants⁷⁻⁹ or those encoding enzymes for modifying membrane lipids^{10,11}, LEA protein¹², and detoxification enzyme¹³. 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 dehydration¹. 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 pathway¹⁴⁻¹⁸. A *cis*-acting element has been identified in the promoter region of the *rd29A* gene and is responsible for dehydration- and cold-induced expression¹⁹. This sequence (TACCGACAT), termed the dehydration-responsive element (DRE), is essential for the regulation of dehydration-responsive gene expression¹⁹ 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 screening²¹. 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 stresses²¹. 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 promoter²⁴ 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 method²⁵.

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* transgene²¹. 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-

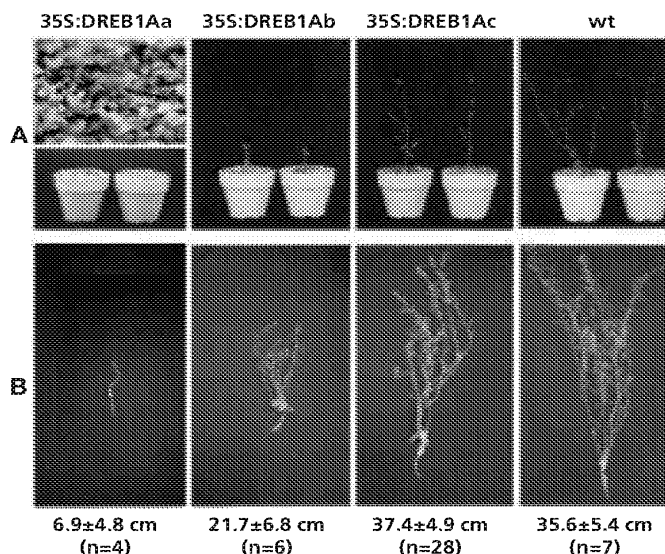


Figure 1. Phenotypes of the 35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac plants in relation to wild-type plants (pBI121). (A) Plants grown for 35 days. (B) Plants grown for 53 days. The average height of each plant line grown for 53 days is indicated under each picture.

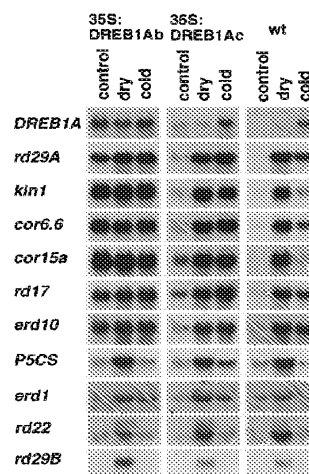


Figure 2. Expression of *DREB1A* target gene mRNAs in 35S:DREB1A transgenic plants and in the wild-type controls. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arabidopsis* plants that had been dehydrated for 5 h (dry), treated at 4°C for 5 h (cold), or left untreated (control). 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.

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 con-

tinued 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-

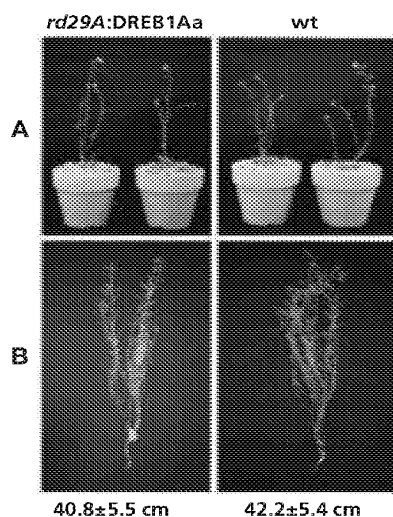


Figure 3. Phenotypes of the *rd29A*:DREB1Aa and wild-type control plants (transformed with the vector pBI121) under control conditions. (A) Plants grown for 30 days. (B) Plants grown for 63 days. The average height of each plant line grown for 63 days is indicated under each picture.

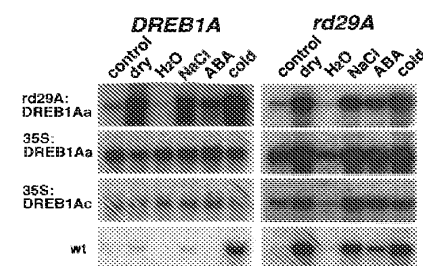


Figure 4. Expression of the *DREB1A* and *rd29A* genes in 35S:DREB1A and *rd29A*:DREB1A transgenic plants. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arabidopsis* plants that had been dehydrated (dry), grown hydroponically in 250 mM NaCl (NaCl), in water (H₂O), or in 100 µM ABA (ABA), or transferred to agar plates at 4°C (cold), or untreated (control). The stress treatments were continued for 5 h.

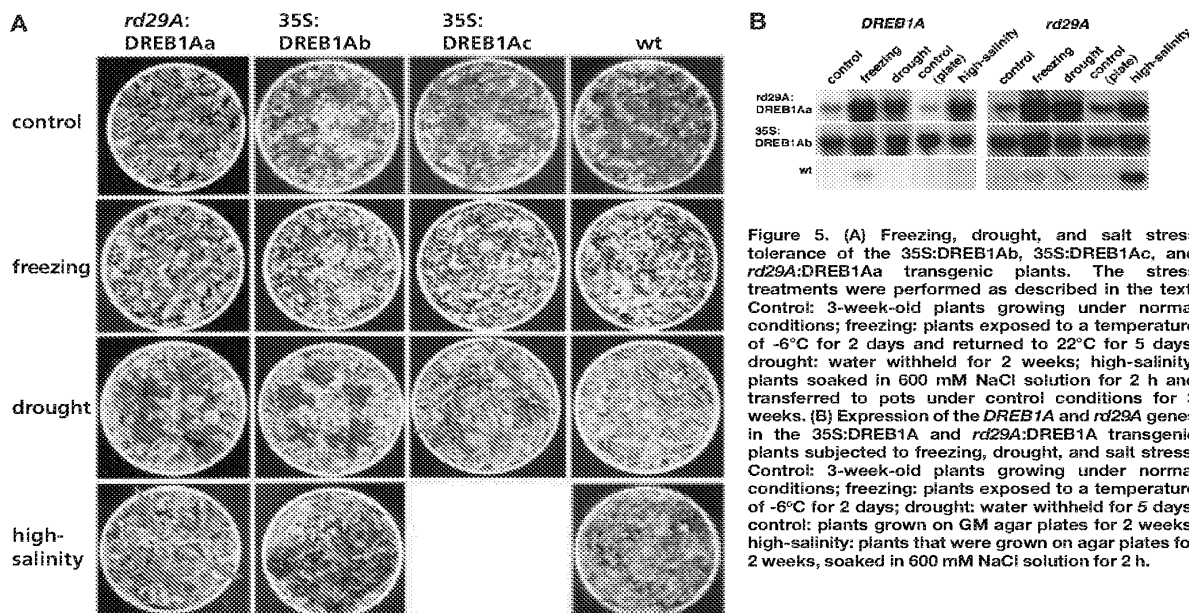


Figure 5. (A) Freezing, drought, and salt stress tolerance of the 35S:DREB1Ab, 35S:DREB1Ac, and *rd29A*:DREB1Aa transgenic plants. The stress treatments were performed as described in the text. Control: 3-week-old plants growing under normal conditions; freezing: plants exposed to a temperature of -6°C 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	%
<i>rd29A</i> :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	%
<i>rd29A</i> :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	%
<i>rd29A</i> :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 proteins¹⁴. The *cor47/rd17* and *erd10* genes both encode group 2 LEA proteins³¹. The *kin1* and *cor6.6/kin2* genes encode proteins that are structurally similar to the alanine-rich antifreeze proteins produced by some fish²⁰. 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 chloroplasts³³, and enhances the freezing tolerance of *Arabidopsis* leaf protoplasts³⁴.

Cold acclimation increases the freezing tolerance of plants². Various genes are induced during cold acclimation, such as *rd29A/rd78/lti78*, *kin1*, *cor6.6/kin2*, *cor15a*, *cor47/rd17*, and *erd10*. The DRE is involved in the induction of these genes in response to low temperatures¹. 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

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^{25,26}. 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 previously²⁰. 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'-AAGCTTGGCCATAGATGCAATTAATC-3' and 5'-AGCTTTTGGAAAGATTTTTTCTTTCCAA-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-NotI-*Sac*I polylinker (Takara, Tokyo, Japan). The constructs were introduced into *Agrobacterium tumefaciens* C58 as described previously¹⁹. 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 method²².

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 previously¹⁹. 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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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 *uidA* 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:*uidA* 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.

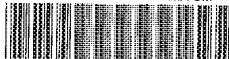
Introduction

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 primarily 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 *uidA* 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*III site to the *Bgl*III 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 oligonuc-

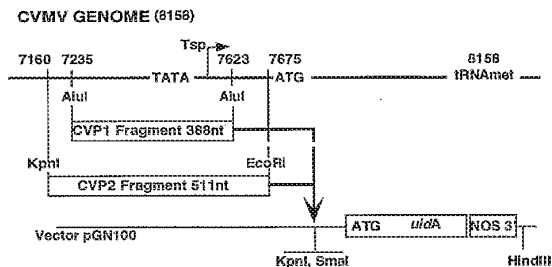


Figure 1. Construction of *uidA* fusion genes. Positions of the promoter fragments are numbered in the CVMV genomic DNA. The CVP1 fragment was isolated using *Alu*I restriction sites while the CVP2 fragment, which included an additional 75 nucleotides at the 5' end and 52 nucleotides at the 3' end, was 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)

leotides 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'-CGGAATTCAAACCTTACAAATTCTCTGAAG-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 μ M 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 $^{\circ}$ C for 5 min then the reaction mixture was denatured at 94 $^{\circ}$ C for 1 min, annealed at 60 $^{\circ}$ C for 1 min and elongated at 72 $^{\circ}$ C for 1 min for each of 15 cycles. Final extension was carried out for 5 min at 72 $^{\circ}$ C. Sequence accuracy of the amplification product was subsequently confirmed by dideoxynucleotide chain-termination sequencing (Sequenase - USB).

Plasmid pLTAB:CVP1 (Fig. 1) was constructed by ligating the *Alu*I CVP1 fragment into *Sma*I site of pGN100, a pUC 119 derived plasmid containing the *uidA*-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 pLTAB:CVP2 plasmid (Fig. 1). The cas-

settes containing the CVMV promoter:*uidA* fusion genes were excised by *KpnI/HindIII* digestion from pILTAB:CVP1 and pILTAB:CVP2 and subcloned at *KpnI/HindIII* sites in the pBIN19 binary vector (Clontech) used for *Agrobacterium*-mediated plant transformation. The plasmid pe35GN contains the enhanced 35S promoter [26] and the *uidA* 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:CVP2 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 h 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 sec-

tions 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'-CGCGATCCAGACTGAATGCCCACAGGCCGTCGAG-3' which is complementary to a region 34 nucleotides downstream of the ATG start codon in the *uidA* gene. The oligonucleotide (20 pmol) was 5' end-labeled using 6 U of T4 polynucleotide kinase (USB) and 7 μ Ci of [γ -³²P]ATP (3000 μ Ci/mmol, 10 μ Ci/ μ l). After the labeling reaction, the primer was purified using Nucletrap Push columns (Stratagene). One-tenth pmol of the labeled primer was mixed with 50 μ g 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 $\times$ Murashige and Skoog vitamins (Sigma), 20 mM CaCl₂, 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

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 $100 \times g$ 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 $100 \times g$) 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 ($5000 \times g$, 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 *uidA* 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 Mco1 1505) grown *in vitro* on medium containing MS salts and vitamins, sucrose 20 g/l, CuSO_4 2 μM , Phytigel 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^{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

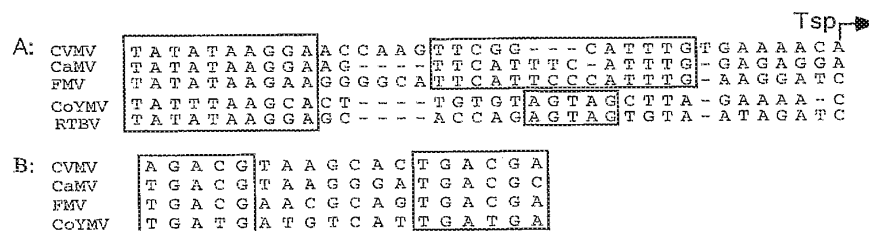


Figure 2. Conserved nucleotide motifs in plant pararetrovirus promoters. A. Comparison of nucleotide sequences from the TATA box to the transcription start site of promoters from plant pararetroviruses [2, 19, 33, 45]. Regions of high homology are framed. Tsp indicates the transcription start point of these promoters. B. Sequence alignment of different *asl* 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 *uidA*-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 *asl* 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: *uidA* 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 transcrip-

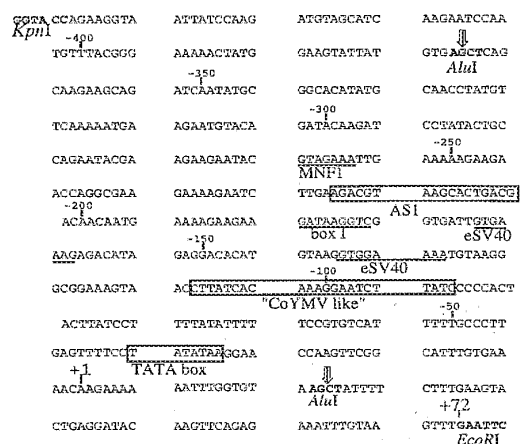


Figure 3. Nucleotide sequence of CVP1 and CVP2 promoter fragments. The transcription start site is designated +1. Consensus TATA box, as *l*-like sequence [29] and the region homologous with the CoYMV promoter [35] are boxed. *AluI* sites (↓) indicate the 5' and 3' ends of the CVP1 promoter. Sequence motifs similar to previously characterized *cis* elements in plant promoters are underlined (motifs with similarities with the box 1 element [13] of the *rbcs* promoter, MNF1-binding site [50], SV40 core enhancer [38] are indicated).

tion 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).

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

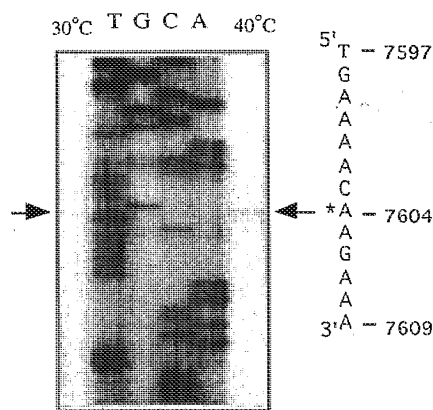


Figure 4. Determination of the transcription start site of the CVMV promoter. Primer extension reactions were carried out as described in Materials and methods. The products of the extension reactions, obtained with two annealing temperatures (30 °C and 40 °C) and reference sequencing reactions of CVP1-*uidA* 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].

used in this experiment, the plasmids pILTAB:CVP1 and pILTAB:CVP2. The plasmid pe35GN, containing the *uidA* sequence under the control of the enhanced 35S promoter (e35S) [26], served as a positive control. Each plasmid was co-introduced 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.

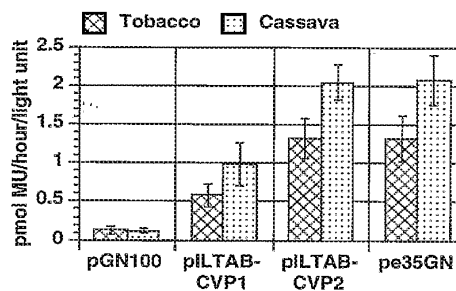


Figure 5. Expression of the CVMV promoters in tobacco and cassava protoplasts. Chimeric *uidA* gene constructs were co-introduced with a plasmid to express luciferase [40] into protoplasts of cassava and tobacco via electroporation. Promoter activity is expressed as a ratio between GUS activity and LUC 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 $\pm$ 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 *uidA* gene. pGN100 contains a promoter-less *uidA* gene.

Expression of the CVMV promoter in transgenic tobacco and rice plants

Seven transformed tobacco lines containing CVP1 promoter-*uidA* 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

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: *uidA* gene. The general expression pattern of the CVP2 promoter-*uidA* 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 p ϵ 35GN (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, mesophyll 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-*uidA* 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 ϵ 35S promoter. Of the two promoter fragments tested in protoplasts, the shorter frag-

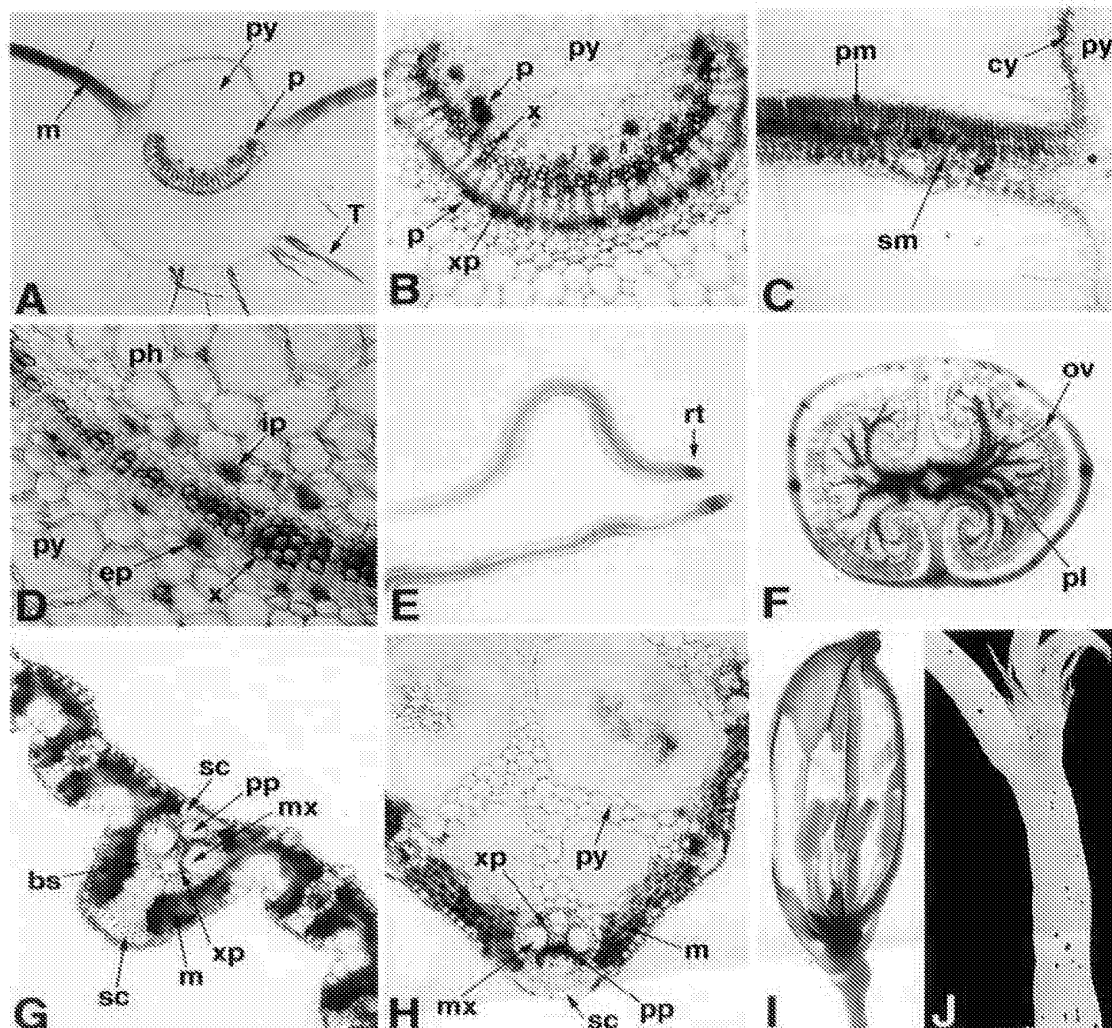


Figure 8. Histochromal localization of GUS expression in transgenic tobacco and rice plants containing CVMV promoter-uidA gene fusion. GUS activity is indicated in the transgenic tissues by an indigo dye precipitate after staining with X-Gluc. Pictures presented are from CVP2-uidA transgenic plants. A. Tobacco leaf section. B. Detail of tobacco leaf section showing vascular tissues of the midrib ($\times 10$). C. Transverse section through tobacco leaf lamina ($\times 30$). D. Vascular tissues in tobacco stem cross-section ($\times 30$). E. Tobacco roots ($\times 10$). F. Transverse section through tobacco ovary ($\times 10$). G. Rice leaf cross-section ($\times 50$). H. Cross-section of a rice leaf sheath ($\times 50$). I. Rice flower split axially and subsequently stained for GUS activity ($\times 10$). J. GUS transient expression on cassava stem from *in vitro* planter ($\times 5$). bs, bundle sheath; cy, chlorenchyma; ep, external phloem; ip, internal phloem; m, mesophyll; mx, metaxylem; ov, ovule; p, phloem; ph, pith; pl, placenta; pm, palisade mesophyll; pp, phloem parenchyma; py, parenchyma; rt, root tip; sc, sclerenchyma; sm, spongy mesophyll; x, xylem; xp, xylem parenchyma.

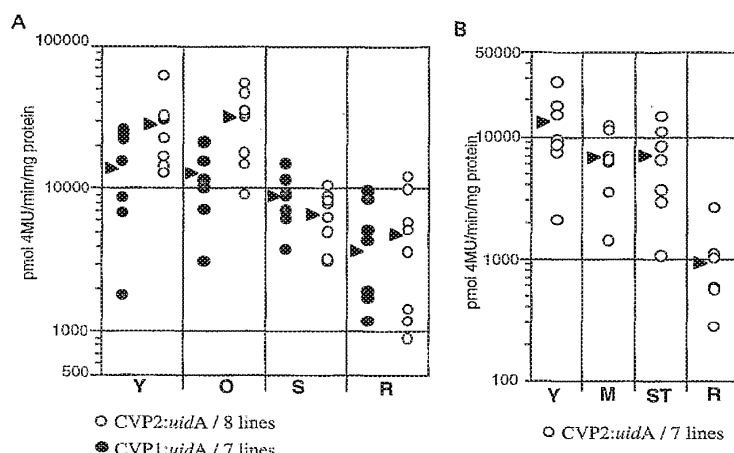


Figure 7. Distribution of GUS activity in different tissues among tobacco (A) and rice (B) transgenic lines. Specific GUS activity is given as picomoles of 4 methylumbelliferone per milligram of protein per minute. Transgenic tobacco lines containing either CVP1 (solid dots) or CVP2 (open dots): *uidA* fusion gene were tested for GUS activity in young leaves (Y / 3 to 5 cm in length), mature leaves (M / 10 cm and up in length), stems (S), and roots (R). Transgenic rice lines tested contained only the CVP2 construct. Samples for protein extraction were taken from mature (5 to 7 weeks old) R1 transgenic tobacco plants grown in a greenhouse. Rice plants used in this study were R0 transformants (2 months old) grown in a greenhouse. In rice, ST refers to leaf sheath explants. Distribution of GUS activity was plotted and is shown for tobacco (panel A) and rice (panel B) plants. Each dot represents a single independent transgenic line. Number of lines tested is mentioned on the figure. Mean level of GUS activity in the different organs and for each construct is indicated by a solid arrow. Logarithmic scale was used to accommodate large variation between lines.

ment CVP1 is approximately two-fold less active than the longer CVP2 fragment. However, both fragments result in the same pattern of expression in transgenic tobacco and plants. Differences in the level of expression observed in protoplasts could be due to a transcriptional enhancer in the 5' region of the larger fragment or to the larger untranslated leader sequence. The latter could be important for greater message stability or translation efficiency. The first 60 nucleotides of the CaMV leader (from +1 to the first ATG) stimulate expression of a downstream gene by about 2-fold [14, 17]. Similar effect has been reported for the untranslated leader of the rice tungro bacilliform virus promoter [11]. However there is limited sequence homology between the CVMV leader and those of the CaMV or RTBV leaders. Analysis of transgenic plants indicate that the CVMV promoter, as is the case with other caulimovirus promoters, is active in all organs, in various cell types and is not dependent on viral trans-acting factors. The CVMV promoter is strongly expressed in vascular tissues, in leaf mesophyll cells, and in the root tips of rice and tobacco plants. However, GUS activity was non detectable in non-chlorophyllaceous cells of tobacco pith and cortical parenchyma. This

could indicate that the CVMV promoter has two major domains of activity, i.e., the vascular elements and the green, chloroplast-containing cells. Analysis of CaMV 35S-*uidA* gene expression in transgenic tobacco and rice plants (not shown) confirmed previous reports [3, 24, 49] and also showed more staining in chlorophyllaceous cells than in cortical parenchyma cells. However, we cannot exclude the possibility that these observations are due to the limitations of the staining assay. Large cells with little cytoplasm (such as parenchyma cells) may appear to contain little or no stain compared with smaller cells with dense cytoplasm. Likewise, cells with different metabolic activities may stain with different intensities.

This study has shown that expression of the CVMV promoter in protoplasts and transgenic plants is relatively similar to that of the 35S promoter. However, the nucleotide sequence of the CVMV promoter has limited homologies with other caulimovirus promoters which may imply differences in the mechanisms of regulation of the promoter. Analysis of the CVMV promoter sequence shows the presence of several motifs that resemble previously identified *cis* elements that are implicated in transcriptional regulation. The pres-

ence of such motifs in the CVMV promoter could explain the pattern of expression in transgenic plants. A 16 bp motif with strong homology with the activation sequence 1 (as1) of the CaMV 35S promoter [29] was identified in the CVMV promoter at nt -203 to -219. This motif is also found in the FMV promoter [46]. A less conserved motif has also been identified in the CoYMV badnavirus promoter [34]. The as1 element, characterized by TGACG direct repeats, binds to the AS1 nuclear factor [16] as well as the cloned TGA1 transcription factor [25]. This motif, in the CaMV 35S promoter, is able to confer expression principally in root tissues [4], but it also plays a more complex role in the regulation of the promoter by interacting synergistically with other *cis*-elements [15, 29]. However, in the CVMV promoter, this as1 motif is located at position -203 to -219 while in the caulimovirus promoters, it is generally closer to the TATA box (-83 to -63 in the 35S CaMV promoter; -57 to -73 in the FMV promoter).

At position -90 to -111 we identified a 22 nucleotide sequence CTTATCACAAAGGAATCTTA TC that is present at the same relative position (-78 to -100) in the CoYMV promoter but not in other plant pararetrovirus promoters. This motif is located in the CoYMV promoter in a region required for expression in vascular tissues [35]. The CVMV promoter also includes the motif AAGATAAGG (-186 to -194) which contains the boxI consensus GATAAG that is present in *rbcS* gene promoters [13]. In addition, the sequence GTAGAAA, identified at position -257 to -263, is identical to the binding site sequence for the MNF1 leaf-specific nuclear factor, found in the PEPC gene promoter as well as in the 35S promoter [50]. These motifs could be involved in the strong gene expression of the CVMV promoters in mesophyll cells. Nucleotides -170 to -130 (Fig. 2) contain two motifs that are similar to the SV 40 enhancer core sequence GTGGAAAG [38]. The relative importance and the biological role of all these motifs in this promoter are being evaluated.

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Purification and Characterisation of the Enantiospecific Dioxygenases from *Delftia acidovorans* MC1 Initiating the Degradation of Phenoxypropionate and Phenoxyacetate Herbicides

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Summary

After cultivation on (*R,S*)-2-(2,4-dichlorophenoxy)propionate, two α -ketoglutarate-dependent dioxygenases were isolated and purified from *Delftia acidovorans* MC1, catalysing the cleavage of the ether bond of various phenoxyalkanoate herbicides. One of these enzymes showed high specificity for the cleavage of the *R*-enantiomer of substituted phenoxypropionate derivatives: the K_m values were 55 μ M and 30 μ M, the k_{cat} values 55 min⁻¹ and 34 min⁻¹ with (*R*)-2-(2,4-dichlorophenoxy)propionate [(*R*)-2,4-DP] and (*R*)-2-(4-chloro-2-methylphenoxy)propionate, respectively. The other enzyme predominantly utilised the *S*-enantiomers with K_m values of 49 μ M and 22 μ M, and k_{cat} values of 50 min⁻¹ and 46 min⁻¹ with (*S*)-2-(2,4-dichlorophenoxy)propionate [(*S*)-2,4-DP] and (*S*)-2-(4-chloro-2-methylphenoxy)propionate, respectively. In addition, it cleaved phenoxyacetate herbicides (i.e. 2,4-dichlorophenoxyacetate: K_m = 123 μ M, k_{cat} = 36 min⁻¹) with significant activity. As the second substrate, only α -ketoglutarate served as an oxygen acceptor for both enzymes. The enzymes were characterised by excess substrate inhibition kinetics with apparent K_i values of 3 mM with (*R*)-2,4-DP and 1.5 mM with (*S*)-2,4-DP. The reaction was strictly dependent on the presence of Fe²⁺ and ascorbate; other divalent cations showed inhibitory effects to different extents. Activity was completely extinguished within 2 min in the presence of 100 μ M diethylpyrocarbonate (DEPC).

Introduction

The degradation of chlorinated/methylated phenoxyalkanoate herbicides is usually initiated by the cleavage of the ether bond of these compounds leading to the formation of respective phenolic moieties and the oxidised alkanoates. The prevailing reaction is catalysed by an α -ketoglutarate-dependent dioxygenase as studied with the purified enzyme from *Ralstonia eutropha* JMP134; α -ketoglutarate is oxidatively decarboxylated

to succinate in this step [1, 2]. The enzyme from the above strain was determined to be highly specific for the phenoxyacetate structure. Genes encoding this type of enzyme, i.e. *tfdA*, were found to be widespread in microbial communities [3–9]. By contrast, there are only a few examples of axenic strains that are able to productively utilise phenoxypropionate herbicides: *Sphingomonas herbicidovorans* (*Flavobacterium* sp.) MH [10, 11], *Rhodospirillum rubrum* sp. P230 [12] and *Delftia* (*Comamonas*) *acidovorans* MC1 [13]. Remarkably, these strains utilise a broader spectrum of phenoxy herbicides; in addition to their ability of degrading the racemic phenoxypropionates, they similarly attack phenoxyacetate derivatives. Again, this reaction proved to be catalysed by an α -ketoglutarate-dependent dioxygenase [14–16].

Preliminary investigations revealed enantioselective properties of the enzymes catalysing the cleavage of the ether bond of the racemic phenoxypropionates as holds for *S. herbicidovorans* MH [14, 17] and *D. acidovorans* MC1 [15]. The enantioselectivity of this reaction is also supported by the fact that a strain of *Alcaligenes denitrificans* is only able to utilise the *R*-enantiomer of 2-(4-chloro-2-methylphenoxy)propionate [18]. These enzymes have, however, not been studied in detail.

The present investigation is aimed at elucidating the enzymatic basis of the broad herbicide consumption profile including the phenoxyacetate derivatives in *D. acidovorans* MC1. Enzymes carrying ether-cleaving activity with respect to the various phenoxyalkanoate herbicides were purified and characterised with regard to their catalytic properties.

Materials and Methods

Cultivation

Strain MC1 was inoculated from individual colonies grown on (*R,S*)-2,4-DP into PYE medium (3 g/l of each peptone and yeast extract and 1.8 g/l of fructose) and propagated in an overnight culture; then it was incubated for a further day in the presence of 100 mg/l of (*R,S*)-2,4-DP. One litre of this culture was used to inoculate a fermenter (INFORS, ALGU 503) operating at a working volume of 4 l. Cultivation proceeded under aerobic conditions at pH 8.0 and 30 °C. The medium was composed of [mg/l]: NH_4Cl , 1520; KH_2PO_4 , 680.5; K_2HPO_4 , 870.9; $\text{CaCl}_2 \times 6 \text{H}_2\text{O}$, 11; $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 142; $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$, 10; $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$, 1.57; $\text{MnSO}_4 \times 4 \text{H}_2\text{O}$, 1.23; $\text{ZnSO}_4 \times 7 \text{H}_2\text{O}$, 0.88; $\text{Na}_2\text{MoO}_4 \times 2 \text{H}_2\text{O}$, 0.5. (*R,S*)-2,4-DP was supplied as the sole carbon and energy source in a fed batch regime by adapting the feed rate to the substrate consumption capacity of the culture. Excess substrate in the medium did not exceed 0.5 g/l. In addition, a trace element solution containing the respective salts in the concentration specified above was continuously fed at a rate of 0.77 ml/h. Biomass increase (OD_{700}) and substrate consumption were monitored off-line. After the cell density had reached an $\text{OD}_{700} = 2$, about 50% of the suspension was harvested. The remaining culture in the fermenter was supplied with an adequate portion of the mineral salt solution and cultivation continued. Cells were harvested by centrifugation at 4 °C at $8875 \times g$. The pellet was washed and re-suspended in 10 mM Tris/HCl buffer, pH 7.5 (buffer A). The suspension was stored at –20 °C pending further use.

Purification

Purification was performed by application of a GradiFrac System including pump P-1 and monitor UV-1 (AMERSHAM PHARMACIA BIOTECH). Unless otherwise indicated, all steps were carried out at 4 °C.

Step 1: Cells were disintegrated using a French Pressure Cell (AMINCO, Silver Spring, USA) by three passages at 140 MPa. Particle-free supernatants were obtained by centrifugation at 4 °C for 25 min at 20,000 × g.

Step 2: Protamine sulphate (1%, pH 7.0) was added to the supernatant to give a final concentration of 0.15%. The solution was stirred for 20 min and separated from the precipitate by centrifugation for 20 min at 20,000 × g.

Step 3: Ammonium sulphate was added by stirring the supernatant from step 2 to give a final concentration of 1.25 M. After stirring for 20 min, the precipitate was removed (20 min centrifugation at 20,000 × g). A further quantity of ammonium sulphate was added to the supernatant to give a final concentration of 2.5 M. After stirring for a further 20 min, the pellet was collected by centrifugation and used for further steps.

Step 4: The pellet was solubilised in a small quantity of buffer A and applied to a Hi Prep 26/10 desalting column (PHARMACIA, Sweden) filled with Sephadex G-25. The column was equilibrated with buffer A. Application of the sample and elution proceeded at a rate of 5 ml/min. The eluate was sampled in fractions of 10 ml, which were monitored for conductivity and protein content.

Step 5: Ion exchange chromatography (IEX) was carried out on a Source30Q (PHARMACIA, Sweden) column (25/14). The column was equilibrated with buffer A. The sample was applied with a rate of 1 ml/min and further treated with buffer A to remove non-bound proteins. The proper elution was started at the same rate by linearly increasing NaCl gradients of varying steepness. The total volume of the gradient solutions amounted to 120–180 ml. Eluates were collected in fractions of 6 ml.

Step 6: Hydrophobic interaction chromatography (HIC) was performed on a Butyl-Sepharose (PHARMACIA, Sweden) column with a bed volume of 20 ml. The column was equilibrated with buffer A containing different initial concentrations of ammonium sulphate. The protein samples derived from each purification step were supplied with ammonium sulphate to reach the desired initial concentration corresponding to the column equilibrium concentration. Desorption was performed by using a linearly decreasing ammonium sulphate gradient at a rate of 1 ml/min. The total volume of the gradient solutions amounted to 180 ml. Eluates were collected in fractions of 6 ml.

Step 7: Gel filtration was performed on a Superdex200 prep grade (PHARMACIA, Sweden) column (100/10). It was equilibrated with buffer A containing 0.15 M NaCl. Before application, the protein solutions were concentrated by precipitation with 3.4 M ammonium sulphate, re-solubilised in a small volume and applied and eluted at a rate of 0.5 ml/min. The eluates were collected in fractions of 10 ml.

Electrophoresis

One-dimensional electrophoresis was carried out with a PowerEase 500 system (NOVEX, San Diego, USA) on 12% Tris glycine precast acrylamide gels (NOVEX). The samples contained 1–2 µg of protein. The running buffer was composed of 2.9 g/l Tris base, 14.4 g/l glycine and 1.0 g/l SDS. The sampling buffer contained 2 ml glycerol, 4 ml 10% SDS [w/v], 0.5 ml 1% bromophenol blue and 2.5 ml 0.5 M Tris/HCl, pH 6.8, in 10 ml distilled water. The gels were treated by 10 mA. Mark 12™ Wide Range Standard (NOVEX) was applied as a reference.

Gels were stained by using the SilverXpress kit (NOVEX) following the instructions of the supplier, or by using Coomassie brilliant blue.

Enzyme Measurement

Ether-cleaving dioxxygenase activity was routinely measured according to FUKUMORI and HAUSINGER [1] by determining the phenolic intermediates liberated in this enzymatic reaction after their reaction with 4-aminoantipyrine. The assay for the enzyme reaction contained 1 mM herbicide (sodium salt), 1 mM α-ketoglutarate, 200 µM ascorbic acid, 200 µM ammonium iron(II) sulphate in 10 mM imidazole buffer (pH 6.75). The buffer was gassed with air at 30 °C for 30 min before adding the individual components. The reaction was performed at 30 °C, started by adding the enzyme at a

concentration of 0.05–0.3 μM (with respect to the monomer). After respective times, usually within a period of 10 min, samples (up to 5) were taken and the enzyme reaction was stopped by adding 50 μl of 20 mM EDTA to 1 ml of the reaction mixture. The phenolic products were determined by adding 100 μl of borate buffer, pH 10 (3.09 g H_3BO_3 ; 3.73 g KCl; 44 ml 1 N NaOH ad 1 l), 10 μl 2% 4-aminoantipyrin and 10 μl 8% potassium hexacyanoferrate III. After 5 min of incubation at 30 °C, the extinction of the samples was measured at 510 nm (U-2000, HITACHI, Tokyo, Japan). The progress curves proved almost linear under these conditions as followed from linear regression giving a measure of confidence of >0.98 with the substrate concentrations tested. The standard deviation of the individual points derived from triplicates amounted to $\leq 8\%$. A comparison of the colorimetric assay with a direct measurement of the reaction by following substrate disappearance and product accumulation via HPLC gave identical rates.

Analytical Methods

Phenoxyalkanoates were determined by HPLC according to [19]. The biomass concentration was measured on the basis of the optical density at 700 nm. An $\text{OD}_{700} = 1$ corresponds to a biomass concentration of about 0.5 g/l dry mass.

Results

Enzyme Purification

Treatment of crude extract in the presence of 0.15% protamine sulphate and precipitation of proteins in the range of 1.25–2.5 M ammonium sulphate kept almost all of the ether-cleaving enzymes of the crude extract detected by measuring activities in the presence of the individual enantiomers of 2,4-DP (Tab. 1) and of 2,4-D (not shown). Application of IEX chromatography separated two protein fractions from each other, which exhibited distinct activity to the various substrates. The fraction eluted at low salt concentrations of around 0.12 M exhibited pronounced activity toward (*S*)-2,4-DP and, in addition, activity with respect to 2,4-D which coincided with the peak at low NaCl concentration. A second fraction found at around 0.35 M NaCl exhibited selective activity by only converting the *R*-enantiomer. Pooled fractions carrying the respective activities were further treated by HIC chromatography. This resulted in a significant purification of the two enzymes (Tab. 1), which were consecutively named the *R*-specific RdpA and the *S*-specific SdpA enzyme, respectively. Again, activity with respect to 2,4-D cleavage coincided with the activity profile for (*S*)-2,4-DP. Characterisation by SDS-PAGE revealed that this purification protocol resulted in a rather pure product with the *S*-specific enzyme (Fig. 1). In contrast, significant impurities were still observed with the *R*-specific enzyme (Fig. 2). Densitometric analysis revealed an enrichment of 80% of the *R*-specific enzyme at this preparation step. Use of gel filtration to remove these impurities was less successful as the activity was drastically diminished (Tab. 1). This step was therefore omitted in further preparations. These impurities did, however, not disturb consecutive kinetic investigations as it was proven that any reaction using the phenolic intermediate as the substrate (chlorophenol hydroxylase) was absent after IEX chromatography. This was verified by keeping product, i.e. dichlorophenol, unutilised within 12 h of incubation in the presence of the respective enzyme

fractions. From the electrophoretic patterns (electropherograms), molecular weights of the (subunits of the) *S*-specific and the *R*-specific enzyme of about 32 kD and 36 kD, respectively, were derived.

Tab. 1. Purification of 2,4-DP/ α -KG dioxygenases from *D. acidovorans* MC1

Purification step	<i>(R)</i> -specific enzyme			<i>(S)</i> -specific enzyme		
	Specific activity [mU/mg]	Purification [-fold]	Total activity [%]	Specific activity [mU/mg]	Purification [-fold]	Total activity [%]
Crude extract	7.2	1	100	6.8	1	100
PS-P	6.7	0.93	93	5.8	0.85	95
AS-P	13.8	1.9	92	11.3	1.7	86
Source 30Q	25.4	3.5	66	101	14.9	65
Butyl-sepharose	172.8	24	45	259	38	8
Gel filtration	154	21.4	1	205	30.2	0.8

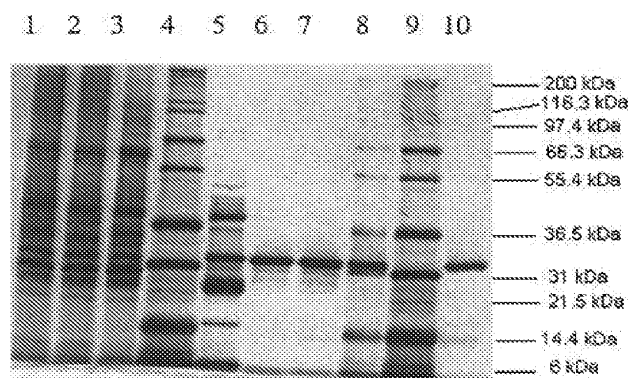


Fig. 1. Purification of *(S)*-2,4-DP/ α -KG dioxygenase from *D. acidovorans* MC1. Protein samples were analysed by gel electrophoresis on a 12% polyacrylamide gel and visualised by silver staining.

1: Crude extract, 2: Supernatant after the treatment with 0.15% protamine sulphate, 3: Fraction precipitated between 1.25–2.5 M ammonium sulphate, 5: Pooled fractions with *(S)*-2,4-DP-cleavage activity after IEX, 6+7: Fractions with *(S)*-2,4-DP-cleavage activity after HIC, 8+10: Pooled fractions with *(S)*-2,4-DP-cleavage activity after GF, 4+9: Molecular weight marker.

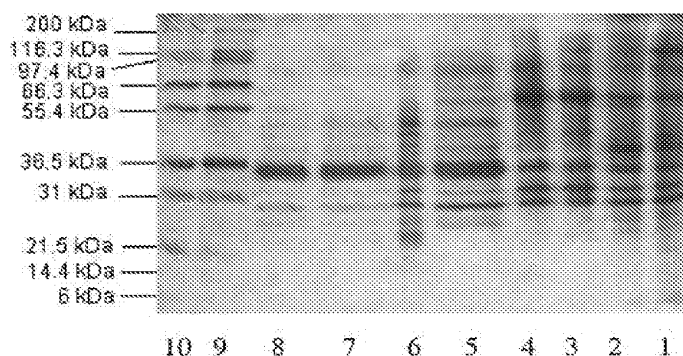


Fig. 2. Purification of *(R)*-2,4-DP/ α -KG dioxygenase from *D. acidovorans* MC1. Protein samples were analysed by gel electrophoresis on a 12% polyacrylamide gel and visualised by silver staining.
1: Crude extract, 2: Supernatant after treatment with 0.15% protamine sulphate, 3+4: Pooled fractions with *(R)*-2,4-DP-cleavage activity after IEX, 5+6: Pooled fractions with *(R)*-2,4-DP-cleavage activity after HIC, 7+8: Pooled fractions with *(R)*-2,4-DP-cleavage activity after GF, 9+10: Molecular weight marker.

The *R*-specific enzyme was found to be stable and did not require supplements when stored at -20° in buffer A. Storage at 4°C resulted in a loss of activity within about 20 days; the enzyme was significantly stabilised under these conditions by adding 1% BSA or 3.4 M ammonium sulphate (Fig. 3). A similar pattern with respect to stability was observed with the *S*-specific enzyme (not shown).

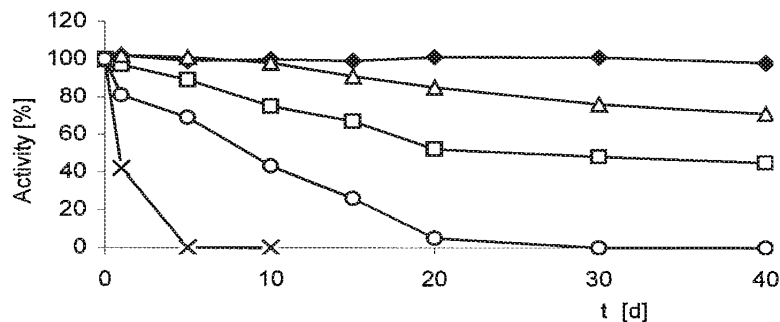


Fig. 3. Stability of the *(R)*-2,4-DP/ α -KG dioxygenase (after HIC) at different storage conditions
(○) 4°C without addition, (×) 4°C after the addition of $50\ \mu\text{M}$ ascorbic acid, (□) 4°C after the addition of 1% BSA, (Δ) 4°C after the addition of 3.4 M ammonium sulphate, (◆) -20°C without addition.

Kinetic Properties

Preliminary investigations were performed in order to elucidate the optimum conditions for the enzyme reaction. The activity of the *R*- and *S*-specific enzyme depended on the presence of both Fe^{2+} and ascorbic acid, as is shown in Figs. 4 and 5. The concentration-dependent activity profiles were characterised by a complex shape (see Discussion). Consecutive kinetic investigations were performed at a Fe^{2+} and an ascorbic acid concentration of $200\ \mu\text{M}$. With regard to the physical parameters, the temperature optimum was determined as $30\ ^\circ\text{C}$ with the *R*-specific and $25\ ^\circ\text{C}$ with the *S*-specific enzyme; the optimum pH value was around 6 with both enzymes. The activity patterns depending on the latter parameters were of similar complexity as indicated above.

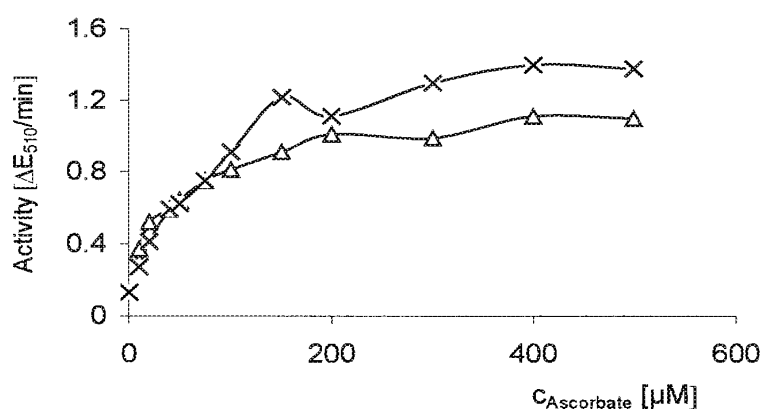


Fig. 4. Effect of ascorbic acid on the activity of 2,4-DP/ α -KG dioxygenases ($\times$) *S*-specific enzyme, (Δ) *R*-specific enzyme.

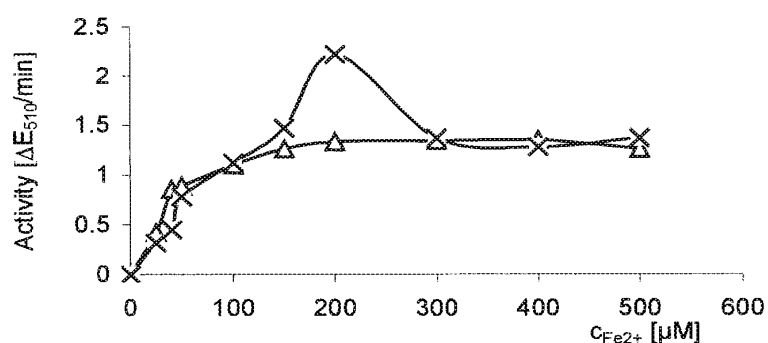


Fig. 5. Activation of 2,4-DP/ α -KG dioxygenases by ferrous ions ($\times$) *S*-specific enzyme, (Δ) *R*-specific enzyme. The enzyme activity was determined using standard assay conditions.

Tab. 2. Substrate specificity and kinetic parameters of (*R*)-2,4-DP/ α -KG dioxygenase

Substrate (concentration range studied)		K_m [μ M]	k_{cat} [min^{-1}]	k_{cat}/K_m	[%]
(<i>R</i>)-2,4-DP	(20–200 μ M)	54.9 ± 4.4	55.2	1	/ (100)
(<i>S</i>)-2,4-DP	(100–5000 μ M)	—	—	—	—
(<i>R</i>)-2,4-MCPP	(10–200 μ M)	30 ± 1.2	34.4	1.15	/ (115)
(<i>S</i>)-2,4-MCPP	(20–600 μ M)	261 ± 40	3.8	0.02	/ (2)
2,4-D	(20–2000 μ M)	1305 ± 120	7.6	0.006	/ (0.6)
2,4-DB	(100–2000 μ M)	—	—	—	—
3-Phenoxypropionic acid	(100–2000 μ M)	—	—	—	—
(<i>R,S</i>)-2-(2,4,5-Trichlorophenoxy)propionic acid	(10–200 μ M)	130.8 ± 9.3	50	0.382	/ (38)
(<i>R,S</i>)-2-(<i>m</i> -Chlorophenoxy)propionic acid	(20–400 μ M)	176.2 ± 4.2	34.4	0.195	/ (19)
(<i>R,S</i>)-2-(4-Chlorophenoxy)propionic acid	(10–200 μ M)	131 ± 0.4	65.2	0.498	/ (50)
α -Ketoglutarate	(2.5–50 μ M)	27.8 ± 3.8	20	0.719	/ (100)
α -Ketobutyrate	(10–5000 μ M)	—	—	—	—
α -Keto adipate	(10–5000 μ M)	—	—	—	—
α -Ketoisovalerate	(10–5000 μ M)	—	—	—	—
α -Ketovalerate	(10–5000 μ M)	—	—	—	—
Pyruvate	(10–10000 μ M)	—	—	—	—

(— = no activity detected)

All experiments were performed at 30 °C in 10 mM imidazol buffer (pH 6) containing 200 μ M ascorbate, 200 μ M $(\text{NH}_4)_3\text{Fe}(\text{SO}_4)_2$, the respective substrates with the concentration ranges indicated, 1 mM of the second substrate [α -KG at variable herbicides; (*R*)-DP at variable keto acids] and enzyme.

Kinetic investigations were aimed at revealing the enzymatic basis of substrate specificity and diversity with respect to the herbicides. The results obtained with the *R*-specific enzyme are summarised in Tab. 2. As expected, the highest activity was obtained with the *R*-enantiomers of the dichlorinated and chloro/methyl-substituted phenoxypropionates taking into account k_{cat} and K_m and the quotient of both parameters. By contrast, very low or no activity was detected in the presence of the respective *S*-enantiomers; 2,4-D was not cleaved at all. This is reflected by both k_{cat} and K_m . Considerable activity was also found with other derivatives of 2-phenoxypropionates including the trichlorinated compound. Although these were applied as the racemates, it is most likely the *R*-enantiomers that were predominantly attacked. (Some effects exerted by the presence of the respective *S*-enantiomers cannot, however, be ruled out under these

conditions. Hence these values are more indicative of the consumption of these compounds rather than usable kinetic constants.) As the second substrate, only α -ketoglutarate served as an oxygen acceptor; alternative substrates were of no effect in a wide range of concentrations tested. It should be noted that by applying variable substrate concentrations similarly complex characteristics were observed as shown in Figs. 4 and 5, the reasons for which are discussed later. Nevertheless, the LINEVEAVER-BURK, HANES and EADIE-HOFSTEE plots exhibited fairly linear dependencies with confidence intervals in most cases of $\approx 95\%$. The kinetic constants derived from such complex characteristics should, however, only be considered as apparent (overall) constants.

The picture was rather different with the *S*-specific enzyme. As expected, pronounced activities were found with the *S*-configuration of 2,4-DP and MCPP. Due to the K_m value, (*S*)-MCPP was the preferred substrate. Most important, however, is the fact that this enzyme is also able to cleave 2,4-D with significant activity (Tab. 3). Again, only α -ketoglutarate served as the second substrate.

Tab. 3. Substrate specificity and kinetic parameters of (*S*)-2,4-DP/ α -KG dioxygenase

Substrate (concentration range studied)		K_m [μ M]	k_{kat} [min^{-1}]	k_{kat}/K_m	[%]
(<i>R</i>)-2,4-DP	(50–500 μ M)	—	—	—	
(<i>S</i>)-2,4-DP	(20–200 μ M)	49 ± 9.1	50	1.02	/ (100)
(<i>R</i>)-2,4-MCPP	(20–1000 μ M)	—	—	—	
(<i>S</i>)-2,4-MCPP	(10–100 μ M)	21.8 ± 5	46	2.11	/ (207)
2,4-D	(50–200 μ M)	122.8 ± 2	36	0.29	/ (29)
2,4-DB	(100–2000 μ M)	—	—	—	
3-Phenoxypropionic acid	(100–2000 μ M)	—	—	—	
(<i>R,S</i>)-2-(2,4,5-Trichlorophenoxy)propionic acid	(100–2000 μ M)	—	—	—	
(<i>R,S</i>)-2-(<i>m</i> -Chlorophenoxy)propionic acid	(20–200 μ M)	11.5 ± 6.4	15	0.13	/ (13)
(<i>R,S</i>)-2-(4-Chlorophenoxy)propionic acid	(10–100 μ M)	68.3 ± 9.8	17	0.25	/ (25)
α -Ketoglutarate	(2.5–50 μ M)	24.1 ± 7.6	1.7	34	/ (100)
α -Ketobutyrate	(10–5000 μ M)	—	—	—	
α -Keto adipate	(10–5000 μ M)	—	—	—	
α -Ketoisovalerate	(10–5000 μ M)	—	—	—	
α -Ketovalerate	(10–5000 μ M)	—	—	—	
Pyruvate	(10–10000 μ M)	—	—	—	

(— = no activity detected)

All experiments were performed at 25 °C in 10 mM imidazol buffer (pH 6) containing 200 μ M ascorbate, 200 μ M $(\text{NH}_4)_3\text{Fe}(\text{SO}_4)_2$, the respective substrates with the concentration ranges indicated, 1 mM of the second substrate [α -KG at variable herbicides; (*R*)-DP at variable keto acids] and enzyme.

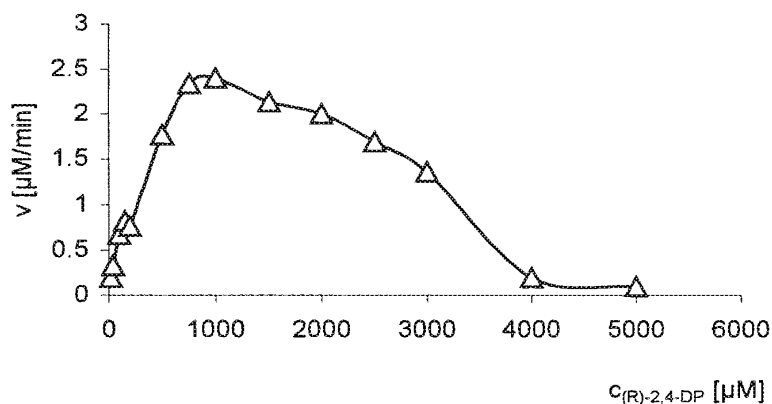


Fig. 6 a. Effect of (*R*)-2,4-DP on the activity of (*R*)-2,4-DP/ α -KG dioxygenase
The assay contained 0.055 μM enzyme.

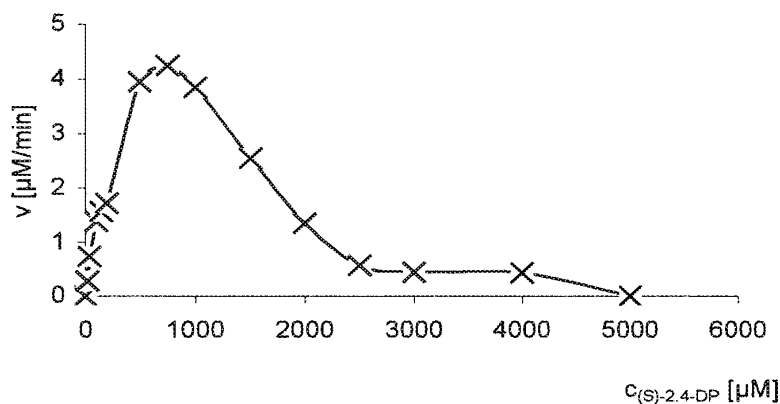


Fig. 6 b. Effect of (*S*)-2,4-DP on the activity of (*S*)-2,4-DP/ α -KG dioxygenase
The assay contained 0.055 μM enzyme.

Both enzymes exhibited excess substrate inhibition. According to the data in Figs. 6a and b, K_i values of about 3 mM with the *R*-specific and of about 1.5 mM with the *S*-specific enzyme can approximately be taken from these data. However, this is only formal and does not reflect the real inhibition pattern, again caused by the complex dependencies on substrate concentration.

Fe^{2+} is essential for the enzyme reaction (Fig. 5), other divalent cations tested such as Cu^{2+} , Mn^{2+} , Mg^{2+} , Zn^{2+} , Ni^{2+} and Co^{2+} could not substitute this ion.

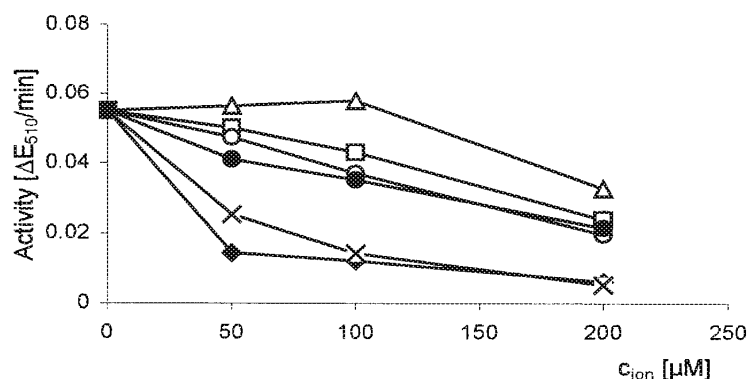


Fig. 7 a. Metal ion-dependent inactivation of (*R*)-2,4-DP/ α -KG dioxygenase
The enzyme activity was determined by using standard assay condition in the presence of various metal ions.

(Δ) Mg²⁺, ($\square$) Ni²⁺, ($\circ$) Mn²⁺, ($\bullet$) Co²⁺, ($\times$) Zn²⁺, ($\blacklozenge$) Cu²⁺.

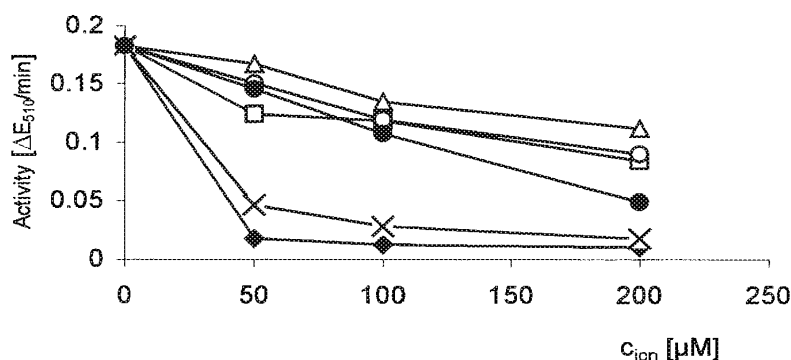


Fig. 7 b. Metal ion-dependent inactivation of (*S*)-2,4-DP/ α -KG dioxygenase
The enzyme activity was determined by using standard assay condition in the presence of various metal ions.

(Δ) Mg²⁺, ($\square$) Ni²⁺, ($\circ$) Mn²⁺, ($\bullet$) Co²⁺, ($\times$) Zn²⁺, ($\blacklozenge$) Cu²⁺.

Moreover, they even behaved in an inhibitory manner. This was most significantly observed with copper and nickel, both of which exhibited, according to the properties shown in Figs. 7 a and b, K_i values of around 50 μ M with the *R*-specific but far less than 50 μ M with the *S*-specific enzyme. The other ions tested exerted weaker effects. In addition, the enzyme was inhibited by DEPC. The application of 100 μ M led to an almost complete loss of activity within 2 min with both enzymes; lower concentrations behaved more gradually (Figs. 8 a,b).

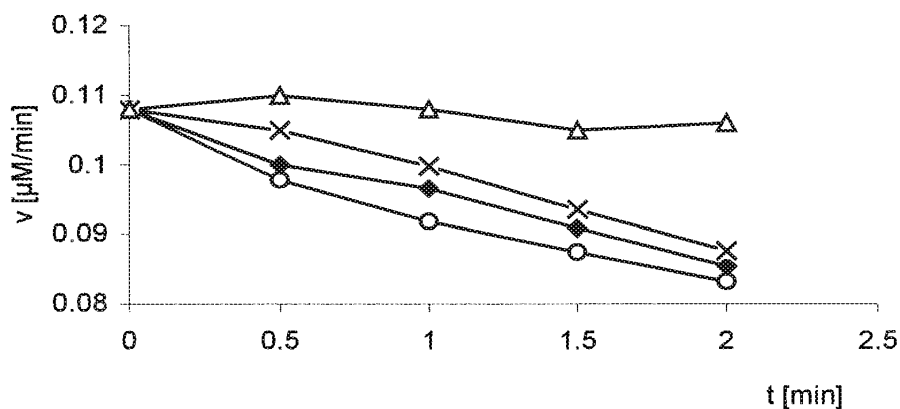


Fig. 8 a. Inactivation of (*R*)-2,4-DP/ α -KG dioxygenase with DEPC
(○) 100 μ M DEPC, (◆) 50 μ M DEPC, (×) 20 μ M DEPC, (Δ) 0 μ M DEPC.

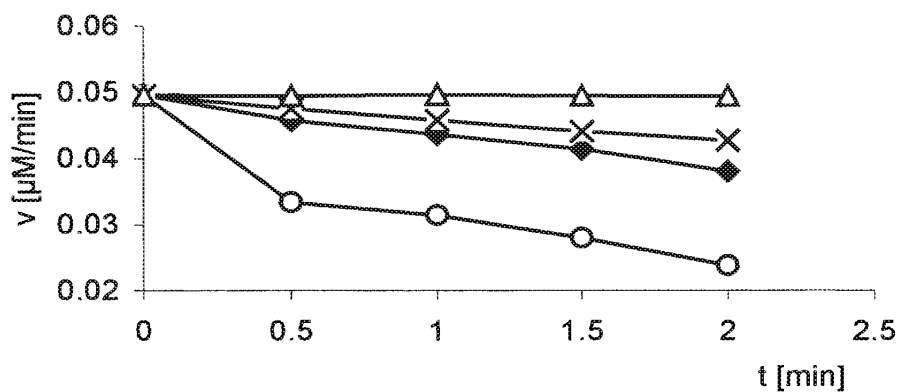


Fig. 8 b. Inactivation of (*S*)-2,4-DP/ α -KG dioxygenase with DEPC
(○) 100 μ M DEPC, (◆) 50 μ M DEPC, (×) 20 μ M DEPC, (Δ) 0 μ M DEPC.

Discussion

The present investigation has clearly elucidated the enzymatic basis for the degradation of enantiomeric phenoxypropionate and the phenoxyacetate herbicides. Two different enzymes with different activity profiles were isolated. One of the enzymes is very specific to the *R*-configuration of the phenoxypropionates, it neither cleaved the *S*-enanti-

mers to a significant extent nor did it utilise phenoxyacetate derivatives (2,4-D). The other enzyme isolated has predominant activity towards the *S*-configuration of the phenoxypropionates; in addition, it exhibits significant activities against 2,4-D. Due to their activities and the predominant substrates utilised, these enzymes should be called (*R*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (RdpA) and (*S*)-phenoxypropionate/ α -ketoglutarate-dioxygenase (SdpA), respectively. The reaction type proved similar to the 2,4-D/ α -ketoglutarate dioxygenase from *Ralstonia eutropha* JMP134(pJP4) [1, 2]; the activity was strongly dependent on Fe²⁺ and ascorbate [16] as was the general case with α -ketoglutarate-dependent dioxygenases [20, 21]. It was inhibited by DEPC, a compound known to be reactive to histidine [22]. Two histidine residues have been shown to be essential components in the active centre of TfdA [24].

Besides the enzymes of strain MC1, there are strong indications of enantioselective enzymes with respect to 2-phenoxypropionates in the case of *S. herbicidovorans* MH. The separation of crude extracts of this strain by SDS electrophoresis revealed distinct protein bands of 32 kD and 34 kD after growth on (*S*)-MCP or (*R*)-MCP, respectively, and of both of these bands after growth on the racemate [14]. This pattern tallies with the molecular weights found for the respective ether-cleaving proteins (subunits) of strain MC1. Moreover, a molecular weight of 32 kD is found with the canonical TfdA protein from *R. eutropha* JMP134 [2], cleaving 2,4-D with high specificity. The application of primers, derived from conserved regions of *tfdA* genes [7], did not result in a specific amplification product in PCR with the genome of strain MC1 [12, 16] and strain MH as the template. This might be expected in agreement with the different substrate specificity found with the various strains (i.e. JMP134 *vs.* MC1). But by comparing strain MC1 and MH, significant differences became obvious, too: whereas strain MH prefers 2,4-D as derived from the maximum growth rate on the various herbicides [17], strain MC1 has a four-fold higher rate on (*RS*)-2,4-DP in comparison to 2,4-D [16]. This means that a similar herbicide consumption profile in these strains must be provoked by a divergent genetic background. This deviates from the experience with *tfdA*: this gene determining 2,4-D degradative specificity is distributed in the microbial world in a highly conserved manner [7]. To complete this picture, a third strain, *Rhodospirillum rubrum* sp. P230, has the same profile of herbicide utilisation as strains MH and MC1. This strain carries TfdA (as followed from sequencing of a respective PCR product), but is nevertheless unable to productively degrade 2,4-D [12, 16]. Very recently, two further strains were described [24] as carrying phenoxypropionate cleavage activity and also giving positive response to *tfdA* probes.

Investigations are in progress to elucidate the molecular structure of both enzymes from strain MC1 and the genes they derive from. It is furthermore apparent from the present results that both enzymes are characterised by complex kinetic behaviour, indicating isoforms of the enzyme. Proteolytic modifications should be ruled out as the cause of this pattern as this was also observed in enzyme preparations carried out in the presence of protease inhibitors. However, two-dimensional electrophoretic separation of the respective enzyme preparations or applying crude extracts revealed protein spots which are indicative of isoforms differing in charge rather than molecular weight [25]. This has to be verified in order to elucidate the structure and catalytic properties of these isoenzymes.

Abbreviations

BSA	Bovine serum albumin
2,4-D	2,4-Dichlorophenoxyacetate
2,4-DB	4-(2,4-Dichlorophenoxy)butyrate
2,4-DP	2-(2,4-Dichlorophenoxy)propionate
DEPC	Diethylpyrocarbonate
HIC	Hydrophobic interaction chromatography
IEX	Ion exchange chromatography
MCPP	2-(4-Chloro-2-methylphenoxy)propionate
GF	Gel filtration

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Isolation of rapeseed genes expressed early and specifically during development of the male gametophyte

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Abstract

A cDNA subtraction and differential hybridization strategy was used to isolate cDNAs expressed early during male gametophyte development in the important crop species *Brassica napus*. Three cDNAs, corresponding to genes highly and specifically expressed at the tetrad and microspore stages, are presented here. The analysis of one of them, named *BnM3.4*, by *in situ* hybridization, showed that it is expressed specifically and at a high level in the rapeseed microspore. The specificity in its profile of expression is most likely transcriptionally controlled as a similar pattern of expression was also observed in *Arabidopsis thaliana* plants transformed by the *BnM3.4* promoter fused to the reporter GUS-coding sequence. The putative *BnM3.4* promoter contains three dispersed copies of a motif described previously in the promoters of several genes expressed in the male gametophyte. The *BnM3.4* gene encodes a predicted novel proline-rich protein of 23.4 kDa which may interact with cytoskeletal components or have a structural role in the cell wall.

Introduction

Pollen development in angiosperm plants occurs in a specialized floral organ, the anther. It starts by a series of mitotic divisions of archeosporial cells from the primary sporogenous layer leading to the formation of meiocytes. Each meiocyte undergoes meiosis to generate a tetrad of four haploid microspores surrounded by a callose wall, whose digestion then liberates them into the anther locule. A complex extracellular matrix composed of intine and exine is built around the male gametophyte after microspore differentiation. An asymmetric cell division of each microspore produces the bicellular pollen grain in which the large vegetative cell encloses the smaller generative cell. This latter cell has a condensed nucleus and a reduced cytoplasm. In the majority of plant species, the second mitotic division of the generative cell occurs after pollination

within the growing pollen tube producing two sperm cells. In some genera such as *Brassica* this second pollen mitosis occurs earlier during pollen maturation. The pollen grain is hence released from the anther locule in a tricellular form. This developmental programme is necessary to prepare the pollen grain for efficient interaction with the stigma, for rapid germination and pollen tube growth, and for successful delivery of sperm cells to the ovules (Bedinger *et al.*, 1994).

Despite the wealth of descriptive studies on the structural and physiological aspects of pollen formation, our understanding of the molecular events remains rather limited. Our main goal is to extend our knowledge on microspore differentiation and development through the identification and characterization of novel genes specifically expressed during early stages of microgametogenesis. Such genes may also provide alternative methods for controlling male fertility in economically important plants, such as in our case *Brassica napus*. To identify genes that might be

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF136223 (*BnM3.4*).

implicated in the developmental process, differential screening of cDNA libraries constructed from whole anthers has been the most commonly used approach. A number of genes have been shown to be expressed in developing pollen, as well as in the tapetum or other sporophytic tissues of the anther (Ursin *et al.*, 1989; Koltunow *et al.*, 1990; Nacken *et al.*, 1991; Theerakulpisut *et al.*, 1991; Paul *et al.*, 1992; Aguirre and Smith, 1993; Robert *et al.*, 1993; Bucciaglia and Smith, 1994; Ross and Murphy, 1996). Some pollen-specific genes expressed after the first mitotic division and presumably playing a role in pollen development, maturation, germination or pollen tube growth have also been described (Hanson *et al.*, 1989; Brown and Crouch, 1990; Shen and Hsu, 1992; Weterings *et al.*, 1992; Brander and Kuhlemeier, 1995; Stanchev *et al.*, 1996; Yu *et al.*, 1998). Finally, so far, a single tobacco gene has been identified that shows a microspore-specific expression (Oldenhof *et al.*, 1996).

We report the isolation of cDNAs from *B. napus* that are expressed during early pollen development. During this work, we developed a novel combined approach by differential screening of a microspore cDNA library with a subtracted probe enriched for microspore-specific sequences. We describe here in detail the characterization of the structure and expression of the *BnM3.4* gene corresponding to the microspore-specific cDNA M3.

Materials and methods

Plant material

Plants of *B. napus* L. cv. Brutor were grown in an open field or under standard greenhouse conditions and were used for RNA and DNA isolation. *Arabidopsis thaliana* (Wassilevskija ecotype) plants used for transformation (Bechtold *et al.*, 1993) were grown in a greenhouse under standard conditions. Transgenic *A. thaliana* T₁ seedlings were selected in a greenhouse on sand, sub-irrigated with water containing Basta herbicide (7.5 mg/l phosphinothricine). Two months later, T₂ seeds were harvested individually. *In vitro* culture of seedlings for segregation analysis was done in a culture chamber on *A. thaliana* medium (Estelle and Sommerville, 1987) containing 5 mg/l of phosphinothricine as selective agent.

RNA isolation and poly(A)⁺ RNA purification

Several grams of rapeseed flower buds from 0.2 mm to more than 4 mm in length were harvested in 4 different classes according to bud length. Bud length was measured from the base to the tip of the outermost sepal. Male gametophytes from graded floral buds were isolated and purified as previously described (Albani *et al.*, 1990) with a few modifications. After disruption of the buds in a blender with a solution of 10% sucrose (pH 7), the resulting suspension was filtered through 80 µm nylon mesh. Only the suspension containing microspores (from 2 to 3 mm buds) was filtered through 45 µm nylon mesh. After two washes, as described by Albani *et al.* (1990), the pellet was frozen in liquid nitrogen and stored at -80 °C if not immediately used for RNA extraction. The purified male gametophytes from the different classes of bud were disrupted by 3 cycles of pressurization (at 110 bar) depressurization in a mini-bomb (Bioblock Scientific, Illkirch, France) in 50% RNA extraction buffer and 50% phenol before performing RNA extraction as previously reported (Dean *et al.*, 1985).

Poly(A)⁺ RNAs were purified from total RNA using the mRNA purification kit from Pharmacia Biotech.

Total RNA was extracted from young rapeseed seedlings, total fertile and male-sterile buds, pistils, sepals and petals, roots, leaves and stems as described above.

cDNA library construction and cDNA subtraction

The procedure used for the isolation of organ-specific cDNA was based on an unpublished protocol from S. Lok and D.C. Baulcombe (Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich, UK). It is the result of several reports on ligation-mediated PCR (Mueller and Wold, 1989), direct incorporation of biotin nucleotide during PCR (Lo *et al.*, 1988) and differential removal of biotinylated DNA by a streptavidin/phenol extraction procedure (Sive and St John, 1988; Wang and Brown, 1991) and was modified as follows.

The blunted 'tracer' cDNA (T) and the 'driver' cDNA (D) were synthesized from 3 µg of poly(A)⁺ RNA from microspores and male-sterile buds (Ogu-INRA) (Gourret *et al.*, 1992), respectively, using a kit from Pharmacia Biotech. An aliquot of the tracer cDNA was ligated to the *EcoRI/NotI* adaptor according to the kit's instructions and inserted into the

*Eco*RI site of λ gt10 and plated on non-permissive *Escherichia coli* strain C600 Hfl⁻ using Gigapack III packaging extract (Stratagene). The titre of the library was estimated at 1.7×10^7 pfu/ μ g of λ gt10 DNA.

A 200 ng portion of T and D cDNA (0.5 to 1 pmol of ends) was ligated to 280 pmol of the T (AATTCGC-CATGGATCTAGACC / pGGTCTAGATCCATG) and D (ATCAGTGATCGGCATGAGCTCG / pCGAGCTCATGCCGA) specific linkers. A 50 ng portion of T and D DNA was then amplified for 12 cycles of PCR for the T (using the top T primer) and 30 cycles for the D using 50 nM Bio-11-dUTP (Sigma) and the top T primer. A 150 ng portion of amplified T was then subtracted with a 20-fold excess of biotinylated D for 24 h at 68 °C. The biotinylated DNA was then removed by the streptavidin/phenol extraction method. The subtraction step was repeated 2 times under the same conditions. 1/6 of the total subtracted tracer-driver (T-D) DNA was then amplified for 35 cycles using the T primer and the result was quantified on a gel. We chose to not directly clone the subtracted product as done by Rubinelli *et al.* (1998) but to use it as a probe to screen the microspore cDNA library in order to compensate the representational bias introduced by PCR.

Differential screening

A 20 ng portion of amplified subtraction product (T-D) and unsubtracted T cDNA were labelled at high specific activity with [³²P] dCTP with an oligolabelling kit (Pharmacia Biotech) (Feinberg and Vogelstein, 1985) and hybridized on duplicate filters (colony/plaque Screen filters, NEN Research Products, DuPont) containing approximately 20 000 plaques of the microspore cDNA library at 65 °C in 6× SSC for 24 h. The filters were washed in three steps of 30 min in 2×, 1× and 0.1× SSC with 0.1% SDS at 65 °C.

Only the clones showing a higher signal when hybridized with the T-D cDNA probe than with the T cDNA probe were selected and isolated. The accuracy of this choice was confirmed by a second round of hybridization realised in the same conditions on filters containing only the selected clones. Positive cDNA clones were purified and λ DNA was extracted as previously described (Albani *et al.*, 1990). cDNA inserts were isolated after *Not*I or *Eco*RI digestion and subcloned in pBluescript SK⁻ plasmid (Stratagene) digested by *Not*I or *Eco*RI.

Northern blot hybridization

A 10 μ g portion of denatured total RNA was loaded in each lane of a 1.5% agarose gel containing formaldehyde and transferred onto Hybond-N membrane (Amersham) according to the manufacturer's protocol. Prehybridization and hybridization were carried out in a buffer containing 5× SSPE, 5× Denhardt's solution, 100 μ g/ml sonicated salmon sperm DNA, 1% SDS and 50% deionized formamide at 42 °C for 6 h and 20 h, respectively. The selected cDNA fragments were gel-purified, labelled with [³²P] dCTP and used as probes for hybridization. The filters were washed at 65 °C in three 15 min steps in 0.1% SDS and decreasing salt concentrations (5×, 2× and 0.1× SSPE).

Genomic DNA library screening

350 000 recombinant clones (average size 15 kb) (three *B. napus* genome equivalents) of a genomic DNA library of *B. napus* cv. Bridger in bacteriophage λ EMBL-3 (Clontech, USA) were plated with *E. coli* LE392 as host. The entire M3 cDNA was used as a [³²P] dCTP-labelled probe for screening. Hybridization and washing were carried out with standard techniques (Sambrook *et al.*, 1989).

Determination of the 5' end of mRNA

The method was based on PCR using the 5'-AmpliFINDER RACE kit from Clontech.

Two antisense primers were designed from the *BnM3.4* coding sequence: P1, 5'-TGATGACTCTAGT TCTGTTGCTGTG-3' and P2, 5'-TGGAAATTCGTTCT GTTGCTGTGACTTTGGATGT-3', and we optimized the annealing temperature at 55°C for the step of PCR amplification. The experiment was performed with 2 μ g of mRNA extracted from *B. napus* microspores, from *B. napus* floral buds and from *B. napus* leaves for the negative controls. Thereafter PCR products were digested by *Eco*RI and cloned into pBluescript SK⁻ for sequencing.

Genomic DNA isolation and Southern blot hybridization

Genomic DNA was purified from seedling leaves using a standard procedure (Dellaporta *et al.*, 1983) followed by standard CsCl gradient centrifugation. Southern analysis was performed by digestion of 10 μ g of genomic DNA, electrophoresis of DNA

fragments on a 0.8% agarose gel and blotting onto Hybond-N membrane using standard methods (Sambrook *et al.*, 1989). The M3 cDNA fragment was gel-purified and labelled with [^{32} P] dCTP as described previously. A Southern blot was hybridized overnight at 65 °C in 6 $\times$ SSC and washed at 65 °C in three 15 min steps in 0.1% SDS and decreasing salt concentrations (6 $\times$, 2 $\times$ and 0.1 $\times$ SSC).

Plasmid construction

Recombinant DNA techniques were carried out as described by Sambrook *et al.* (1989) to construct a transcriptional fusion bringing the expression of the *uidA* (commonly named *gus*) reporter gene under the control of the *BnM3.4* promoter in binary vector.

A 'promoterless' *gus* gene cassette, containing the *gus* coding sequence (Jefferson *et al.*, 1986) and the nopaline synthase polyadenylation site (*nos-ter*; Bevan *et al.*, 1983) was cloned into pBluescript SK $^{--}$ (Stratagene, USA), leading to pAF10pt. A 2056 bp *Bam*HI-*Nsp*V fragment (Figure 3) from the genomic clone *BnM3.4* was cloned into *Bam*HI-*Cla*I sites of the plasmid pAF10pt, generating pJD51. The chimaeric gene was then excised from pJD51 and introduced into the binary plasmid pEC2 (Cartea *et al.*, 1998) in inverse orientation with respect to the Basta resistance gene, generating pJD101. The promoterless *gus* gene cassette was also transferred into pEC2 to generate the negative control binary plasmid pAF100. The binary plasmids pAF100 and pJD101 were introduced into *Agrobacterium tumefaciens* strain C58C1 (Koncz and Schell, 1986) by electroporation (Nagel *et al.*, 1990; Singh *et al.*, 1993). The recombinant genes were introduced into *A. thaliana* by *in planta* infiltration (Bechtold *et al.*, 1993).

In situ hybridization

The *BnM3.4* coding sequence was cloned in both orientation in pGEM-3Zf(+) (pJD6 and 7). After *Sma*I linearization, these two plasmids were used to synthesize digoxigenin-11-rUTP-labelled probes using the Riboprobe Combination System T7 kit from Promega.

B. napus floral buds were fixed in 4% formaldehyde, embedded in wax, and 8 μ m-sections were prepared for *in situ* hybridization according to Jackson (1991).

Full-length RNA probes were alkaline-hydrolysed to 150 nt fragments and hybridized to sections at a concentration of 0.5 ng/ml per kb of probe. Hybridization was carried out in 210 μ l for a sandwich of

two slides at 50 °C in 50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 0.3 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA, 1 mg/ml yeast transfer RNA. Slides were washed in several baths of 0.2 $\times$ SSC at 55 °C for 1 h, followed by two rinses of 5 min each with 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (NTE buffer), and treated with 20 mg/ml RNase A in this buffer at 37 °C for 30 min. The slides were then washed again in NTE buffer and 0.2 $\times$ SSC as described above, and finally washed in PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7) for 5 min. Immunological detection of the hybridized probe was carried out as described in the Boehringer digoxigenin-nucleic acid detection kit with some modifications. Slides were incubated with gentle agitation for 1 h in 0.5% blocking agent (Boehringer) in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) followed by 1 h in 1% bovine serum albumin, 0.3% Triton X-100 in buffer 1 (buffer 2). This was followed by a 1 h incubation in dilute antibody conjugate (Boehringer) (1:1250) in buffer 2 and 4 washes of 15 min each in buffer 2. Slides were briefly washed in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂ and incubated for 12–48 h in 0.34 mg/ml nitroblue tetrazolium salt and 0.175 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂. The colour reaction was stopped with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and sections were passed through an ethanol series before mounting in mounting medium from Sigma.

Histochemical GUS assays

Histochemical assays for GUS activity were conducted according to the protocol described previously by Jefferson *et al.* (1987) with some modifications. Fresh tissue samples were fixed in a solution containing chloroform, 95% ethanol and water in proportions 3:6:1 and 0.1% Triton X-100 under vacuum (-93.3 kPa) for 1 min. Then, tissue samples were washed twice in 50 mM potassium phosphate buffer, pH 7.0. GUS staining was performed by vacuum infiltration (3×10 min at -93.3 kPa) in the GUS stain solution (Jefferson *et al.*, 1987) and by incubating at 37 °C overnight. The plant material was then cleared by rinsing with 70% ethanol and the samples were examined and photographed under a Diaplan type 307-148.002 microscope (Leitz, Wetzlar, Germany).

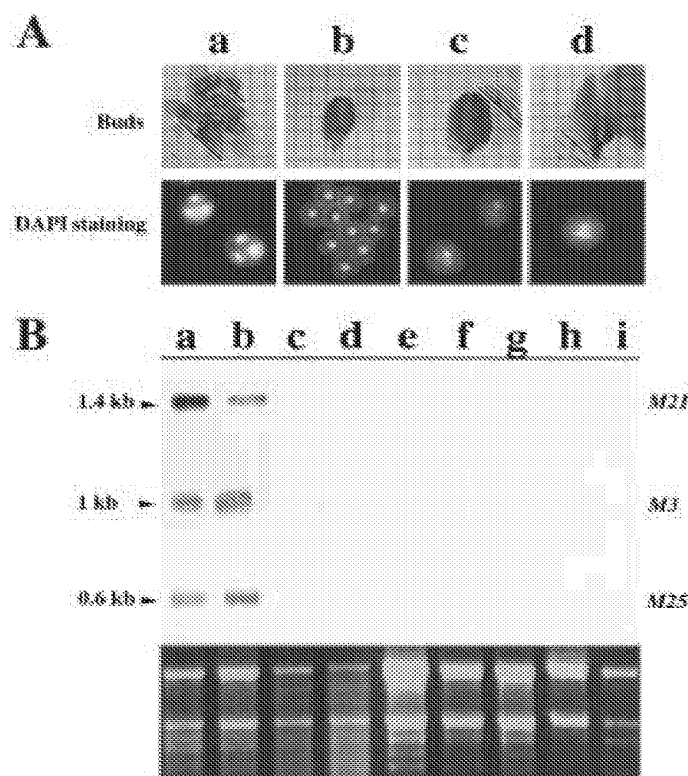


Figure 1. Correlation between the size of rapeseed flower buds and microgametophytic stage of development. Expression pattern of the M21, M3 and M25 clones isolated by subtraction and differential screening of the microspore cDNA library. A. DAPI staining of the four different developmental stages during male gametogenesis (in the lower part) and the corresponding buds (in the upper part). a, DAPI-stained tetrads and earlier stages (not shown) contained in buds of length strictly below 2 mm; b, microspores contained in buds of 2–3 mm; c, bicellular pollen contained in buds of 3–4 mm; d, tricellular pollen contained in buds of 4.5 mm and more. Each square is 1 mm $\times$ 1 mm. B. Northern blot experiments hybridized with M21, M3 and M25 cDNA clones as probes. Total RNA (10 μ g) from (a) meiocyte/tetrads, (b) microspores, (c) young (binucleate) and (d) mature (trinucleate) pollen, (e) sepals, (f) petals, (g) wild type and (h) CMS buds, and (i) *in vitro* grown seedlings were loaded on the gel. The picture of the corresponding ethidium bromide stained gel is presented underneath.

The pistil length was used as the unique criterion for identification of the stages of microsporocyte development in *A. thaliana*. Based on results of Bowman (1994), the correlation between the pistil length (pl) and the stages of microsporocyte development was further studied (D. Vezon, personal communication) and we found that a pl of 0.3–0.4 mm corresponds to meiosis; pl 0.5 mm corresponds to the end of meiosis and tetrad microspores; pl 0.75 corresponds to free microspores; pl 1.25 mm corresponds to the first pollen mitosis and bicellular pollen; pl 1.50 mm corresponds to the second pollen mitosis and tricellular pollen.

Results

Isolation of B. napus cDNAs encoding sequences expressed early during pollen development

Cytological studies (Scott *et al.*, 1991) have shown that the stages of male gametogenesis within the anther are highly correlated to bud length and that gametogenesis is synchronised in all anthers of one flower bud.

The cytological studies allowed us to establish that, in the growth conditions we used, rapeseed buds of length strictly below 2 mm contained mainly meiocytes and tetrads, 2 to 3 mm long buds contained

M3		80
M3.21	TCCTGCTATG ATTTTCTTCA TAAGATGTGT CACATCCAAA GTACACAGAA CAGAACTAGA GTCATCAACT AACCAAGAGC	
M3		160
M3.21	TCTTCTATTC GCGGCACFTG CCTGCTTTTC ACCCCAAGCC ACATTGGGCG TTCTGTGGCT CCGGAAAAGC CTTCCTGCA	
M3		240
M3.21	GGCCACTTCC GACCAACTCC GTTCCATCTG CCACAGGAAG TCACCAGATG CTGTGCGGAC AAGAAGGAGG TAGGTACATG	
M3		320
M3.21	TTTGTATGAT ATCGTTGAGA CTTCCTTCAC CAGGAAAAGC GTTATTTGGAT CGGAATGTTG CCGCGGATC AAGAAGATGA	
M3		400
M3.21	ACAAAGATG TGAGAAGACC GTCTTTGGAT CTTCCTTCAC CCGCTTCTTG ACCGGCTATG TCAAGCTACA TTGCTCCACC	
M3		480
M3.21	GTGTGTGGAT CTACTTCACC TCCTCCTTCA CAGGCTCCTT <u>TACATGCTCC</u> <u>TTCTTCACAG</u> <u>GCTCCTTCAC</u> <u>ATGCTCCTTC</u>	
M3	ACATGCTCCT TCACAGGCTC <u>CTTTAAATGC</u> <u>TTTTTTAAAT</u> GCTCCCTTAC ATGCTCCTTT ACAATGCTCT TCACAGGCCC	560
M3.21	-----C ATGCTCCTTC <u>ACAGGCTCCT</u> <u>TTACATGCTCC</u>	
M3	CTTCACAGGC CCCTTCACAG GCGCCTTTAC ATGCTCCTTT ACTGCCCTCT TCGCAGGCTC CTTCACCGGC TCACATGA-TT	640
M3.21	<u>CTTCACAGGC</u> TCCTTTACAT GCGCCTTCAC AGGCTCCTTT ACTGCCCTCT TCACAGGCTC TCCACCGGC TCACATGATTT	
M3	TAGCTATTTG ATAGAATTAC TCAAGTAATG ATGCCCTAGG GAGTTTGAGT TTTTTCGGTG TTTTAAAGTT TTGTGTTTAT	720
M3.21	TAGCTATTTG TTAGAATTAT TCAAGTGTG ATGCCCTAGG GAGTTTTAGG TTTT-TCTTG TTTTAAATTT TTGTGTTTAT	
M3	TTTGAGAAAA CCGTCTTTGG ATTTTAACTT CACTTTGATT TTTTCCCTTA TACAATTTAA ATTTAGAGTT TACTTATTAA	800
M3.21	TTTGAGAAAA CCGTCTTTGG ATCTTAACTT CACTTTGATT TTTT-CCTTA TACAA.....	
M3	TTTTATAAAT TAGATGGTAC TAAGTTTTTA TCATAATAAA A	841
M3.21		

Figure 2. Comparison of M3 and M3.21 cDNA sequences. Numbers at the right margin refer to the nucleotide position. Dashed lines indicate gaps and underlined sequences represent imperfect repeated motifs found in the M3 and M3.21 sequences. The stop codons are shown in bold type.

mainly microspores, 3 to 4 mm long buds contained mainly bicellular pollen grains and buds of 4.5 mm and more in length contained mature tricellular pollen grains (Figure 1A). RNAs were prepared from male gametophytes purified from buds at these four stages. To achieve this we had to develop a new technique (see Materials and methods) to lyse the microspores which have extremely resistant cell walls. To obtain a driver for the subtraction procedure, we isolated RNA from whole male-sterile flower buds (Ogu-INRA). These buds are identical to those of wild type except that male gametophyte development is arrested before the tetrad stage and consequently they do not contain any microspores (Gourret *et al.*, 1992).

Poly(A)⁺ RNAs from microspores were converted to cDNA, of which a fraction was used to construct a cDNA library. To generate probes enriched for microspore-specific transcripts, another fraction of the above microspore cDNA was then subtracted sev-

eral times by hybridization with cDNA made from male-sterile buds (see Materials and methods). We then screened the microspore cDNA library using subtracted and primary probes. After two rounds of such successive screens, we retained 32 potential candidates that showed increased hybridization to the subtracted probe with respect to the unsubtracted microspore cDNA probe.

The expression pattern of selected clones was compared in more detail by slot blot (data not shown) and by northern blot analyses with total RNA isolated from the four same developmental stages of male gametophyte as above, entire wild-type and male-sterile buds, sepals, petals and *in vitro* grown seedlings or leaves. These expression studies allowed us to classify these genes into four groups.

The first group consists of 13 clones whose expression is strictly confined to the male gametophyte and high in the microspore. The second group contains 13

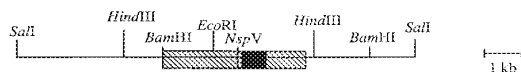


Figure 3. Analysis of the *BnM3.4* gene. Partial restriction map of the *BnM3.4* genomic clone. The striped box represents the sequenced region of the *BnM3.4* genomic clone; the black one the coding sequence of the *BnM3.4* gene. The two *SalI* sites border the 10 kb insert.

clones whose expression is high in the microspores but not restricted to the male gametophyte since they are also expressed in other sporophytic tissues. The third and fourth groups correspond respectively to 2 clones having a weak expression restricted to microspores and 4 clones showing weak expression in all tissues tested.

We characterized further three cDNAs belonging to the first group of genes expressed early and specifically during male gametophyte development. Northern blot analyses of these clones are shown in Figure 1B. The M21 gene detected a mRNA of 1.4 kb (encodes potentially a novel protein), which is expressed at the meicyte and tetrad stages but its mRNA level remains high in the microspore. The M25 cDNA detected a 0.6 kb mRNA (encoding a putative SKP1-like protein (Connelly and Heiter, 1996; Krek, 1998)), mainly in the microspore but expression was also detected at meicyte/tetrad stages and in bicellular pollen. The M3 cDNA detected a 1.0 kb mRNA in microspores but expression was also evident in meicyte/tetrad stages. We have chosen to focus on this particular clone for further studies because extensive hybridization studies using a variety of sporophytic tissues (data not shown) suggested that the M3 mRNA is highly early stage microspore-specific.

Analysis of the nucleotide sequences of M3 and M3.21 cDNAs

The M3 cDNA has a length of 497 bp. It was smaller than the 1.0 kb mRNA detected on northern blots, so M3 is likely to be a partial cDNA. A second screening of the microspore cDNA library was performed using M3 as probe. A longer but still partial clone, named M3.21, of 674 bp was then isolated. The expression pattern of the M3.21 cDNA was checked by northern blot (data not shown) and appeared to be identical to the M3 pattern. Both cDNA clones, M3 and M3.21, were subcloned into pBluescript SK⁺ and sequenced. Alignment of their nucleotide sequences shows that the M3 cDNA differs essentially from M3.21 by an

insertion of 99 bp constituted by three repeats of the same motif (Figure 2).

M3 cDNA and the corresponding BnM3.4 genomic clone: DNA sequence comparisons and analyses

The M3 cDNA was used as a probe to screen a *B. napus* genomic DNA library in order to isolate the corresponding gene. One of the isolated clones, BnM3.4, with an insert size of 10 kb, was selected for further characterization (Figure 3). A 2.9 kb sequence surrounding the *BnM3.4* gene was deposited in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number AF136223. Two putative in-frame translation start codons, separated by 114 bp and leading to 2 ORFs of 712 bp and 598 bp, were detected (respectively at 1971 and 2085 nucleotides from the start of the sequence). Comparison of the M3 cDNA sequence with the sequence of the shortest ORF of the genomic clone revealed 1 difference out of 293 bp of aligned coding sequence and 7 differences out of 203 bp of aligned 3'-downstream region. These sequence differences between genomic and cDNA clones were repeatedly found in independent sequencing experiments and might be due to allelic variations between the different *B. napus* varieties used in the construction of the cDNA and genomic libraries. No intron was detected in the region covered by the cDNA and no intron/exon junctions were found in the rest of the ORF using a splice site prediction program (Hebsgaard *et al.*, 1996). A putative polyadenylation signal (Joshi, 1987b) was found 197 bp downstream of the stop codon. Because we are ultimately interested in the regulation of pollen and anther-specific genes, we first determined by 5' RACE which of the two potential start codons in M3/M3.21 are used exclusively or preferentially. Sequences of 12 PCR fragments revealed two groups of products. The first group contained seven sequences identical to the *BnM3.4* gene (PCR1 in Figure 4). The longest PCR product showed that the transcription start of the *BnM3.4* gene is a G (underlined on Figure 4), localized between the two potential translation starts. Primer extension experiments (data not shown) indicated approximately the same nucleotide for the transcriptional start site. The length between the transcriptional start site and the second ATG (64 bp), is in agreement with the predominant length of leader sequences (40–80 nucleotides) in plant genes (Joshi, 1987a). Moreover, the context of the second ATG agrees well with the consensus sequence (AAorCAAUGGC) proposed for

BnM3.4	-21	AACCCCTAAGC	CCTTACCAC	CGATAACCAT	CAAAACTTTT	CTTCTCGTTT	29
PCR1				.GATAACCAT	CAAAACTTTT	CTTCTCGTTT	
PCR2			ACT	CAATAACCAT	CAAAACTTTT	CTTCTCGTTT	
BnM3.4		CGCTAACTCA	AGGCTTCGAA	AAGTAAAAA	AACAATGAAG	AATGTCACAC	79
PCR1		CGCTAACTCA	AGGCTTCGAA	AAGTAAAAA	AACAATGAAG	AATATCACAC	
PCR2		CGCAAACTCA	AAGCTTCAAA	AAGT---AAG	AACAATGAAG	AATGTCTCAC	
BnM3.4		TGTTCTTTC	TATGATCCTC	TTCTTAAGCT	GTGTCACATC	CAAAGTTACA	129
PCR1		TGTTCTTTC	TATGATCCTC	TTCTTAAGCT	GTGTCACATC	CAAAGTTACA	
PCR2		TGTTCTTTC	TATGATCCTC	TTCTTAAGCT	GTGTCACATC	CAAAGTCACA	
BnM3.4		GCAACAGAAC	TAGAGTCATC	AACTAACCAA	GAGCTCTTCC		169
PCR1		GCAACAGAAC					
PCR2		GCAACAGAAC					

Figure 4. Alignment of the *BnM3.4* gene sequence and the longest representatives of the two types of products PCR1 and PCR2 from the 5' RACE PCR on mRNA from *B. napus* microspores. Dashed lines indicating gaps and nucleotide differences are reported in bold type in PCR2. The transcription start site (G) is underlined (in *BnM3.4* and PCR1) and the ATG codon is in bold type.

dicotyledon nuclear genes by Joshi *et al.* (1997). A clear TATA box sequence (TATATATA) was identified 31 nucleotides upstream of the start of transcription (Messing *et al.*, 1983). The second group of 5' RACE PCR products (PCR2 in Figure 4) presents several nucleotide differences with respect to the first group. The detection of this second group proves that a second gene closely related to *BnM3.4* exists and is expressed in the microspore at approximately the same level as *BnM3.4*. It should not correspond to allele variation of *BnM3.4* because cv. Brutor used to make the cDNA library and the 5' RACE experiments is an inbred line. This gene may correspond to the M3.21 cDNA but M3.21 is too short at its 5' end to enable us to compare it to the PCR2 sequences and determine whether they come from the M3.21 type mRNAs. To ascertain this, a Southern blot analysis was performed on total DNA extracted from *B. napus* cv. Brutor and digested by *Bam*HI, *Eco*RI and *Hind*III. The DNA gel blot was hybridized with ³²P-labelled M3 cDNA at high stringency. As shown in Figure 5, in addition to the 5 kb *Hind*III and 5.5 kb *Bam*HI fragments corresponding to the *BnM3.4* genomic clone, the *B. napus* genome contained other fragments which were clearly recognized by the M3 cDNA probe, consistent with the idea of at least one other expressed homologue in *B. napus*. These homologous genes will be called the *BnM3* family.

Characteristics of the putative *BnM3.4* protein

The *BnM3.4* gene encodes a putative 218 amino acid protein with a molecular mass of 23.4 kDa and a predicted pI of 8.8. The predicted *BnM3.4* protein shows limited similarity with two potential *A. thaliana*

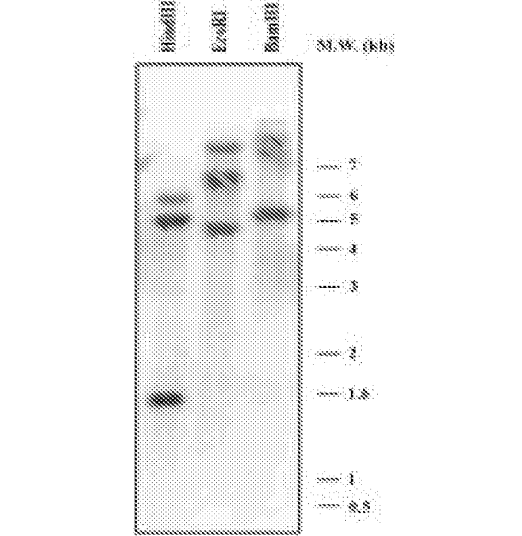


Figure 5. Southern blot analysis of genomic DNA from *B. napus* (cv. Brutor). A 10 µg portion of genomic DNA was digested with *Bam*HI, *Eco*RI and *Hind*III. Hybridization was performed with ³²P-labelled M3 cDNA as probe. DNA size standards (in kb) are shown to the right of the figure.

products of genomic clones MKP11 (accession number AB005238) and K19P17 (AB007644). The N-terminal 152 aa of *BnM3.4* is 55% identical (69% similarity) to MKP11, while the carboxyl portion of *BnM3.4* is 45% identical (63% similarity) to K19P17. These two translated sequences do not correspond to two exons of the same gene since they are separated

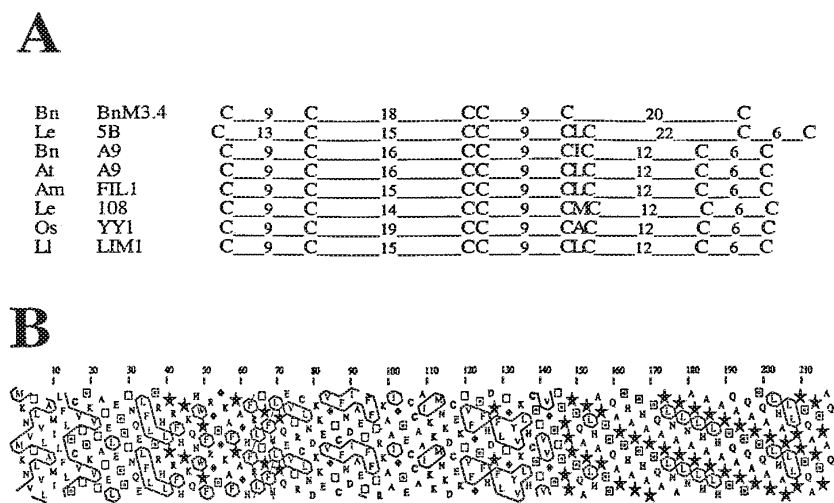


Figure 6. BnM3.4 protein characteristics. A. Cysteine pattern alignment of the BnM3.4 protein with sequences of seven stamen-specific genes. The number of amino acids between cysteine residues is given, but the spacing is not proportional. Le 5B, *Lycopersicon esculentum* TomA5B (Aguirre and Smith, 1993); Bn A9, *Brassica napus* A9; At A9, *Arabidopsis thaliana* A9 (Paul *et al.*, 1992); Am FIL1, *Antirrhinum majus* FIL1 (Nacken *et al.*, 1991); Le 108, *Lycopersicon esculentum* 108 (Chen and Smith, 1993); Os YY1, *Oryza sativa* YY1 (Hihara *et al.*, 1996); Li LIM1, *Lilium longiflorum* LIM1 (Kobayashi *et al.*, 1994). The amino acid sequences were deduced from DNA sequences. B. HCA (Hydrophobic Cluster Analysis) representation of the BnM3.4 protein. Amino acids are represented with one-letter symbols except for: proline (stars); glycine (diamonds); threonine (open squares); and serine (squares with dot). Hydrophobic clusters are endorsed by a black line.

by 16 Mb on the chromosome. The functions of these two putative *A. thaliana* gene products are not known.

The putative BnM3.4 protein, however, has three recognizable structural domains. The residues from 1 to 23 are hydrophobic, characteristic of a signal peptide, and a cleavage site between the alanine at position 23 and the threonine at position 24 can be predicted according to the rules of von Heijne (Nielsen *et al.*, 1997). From these features, the protein may be secreted and this N-segment may help the protein to cross or to interact with the membrane. The central part of the protein (residues 25–143) is hydrophilic. The spacing of six particular cysteines in this segment resembles that found in various stamen-specific proteins: TomA5B (Aguirre and Smith, 1993), FIL1 (Nacken *et al.*, 1991), A9 (Paul *et al.*, 1992), 108 (Chen and Smith, 1993), YY1 (Hihara *et al.*, 1996) and LIM1 (Kobayashi *et al.*, 1994). The possible relationship among these cysteine-rich proteins and the BnM3.4 protein is best illustrated by showing only their pattern of cysteine residues (Figure 6A). Although the BnM3.4 protein presents a shorter pattern than the others, the position and spacing of the cysteine residues are very similar.

The C-terminal domain consists of 14 repeats related to the motif P-[SL]-[HNQ]-A. Analysis of this domain by the Hydrophobic Cluster Analysis program (available at: www.lmcp.jussieu.fr/~soyer/www_hca/hca-form.html) suggests that the proline residues in the motifs could form the backbone of a rigid helical structure (Figure 6B).

In situ analysis of the expression of the BnM3 genes family

The pattern of *BnM3* expression during the four stages of gametogenesis (see above) was examined in bud tissues of *B. napus* by *in situ* hybridization using the *BnM3.4* antisense transcript as a probe. As at least two homologous genes are present in the *B. napus* genome, the hybridization pattern may reflect the expression pattern of (at least) these two genes. The *BnM3* transcripts were not detectable in anthers with pollen mother cells undergoing meiosis (data not shown), nor in tetrads (Figure 7A), nor in bicellular pollen (Figure 7G), nor in tricellular pollen (Figure 7I). However, at the developmental stage where rapeseed buds are 2.5 mm, a strong hybridization signal was detected in the microspore (Figure 7C). At high magnification

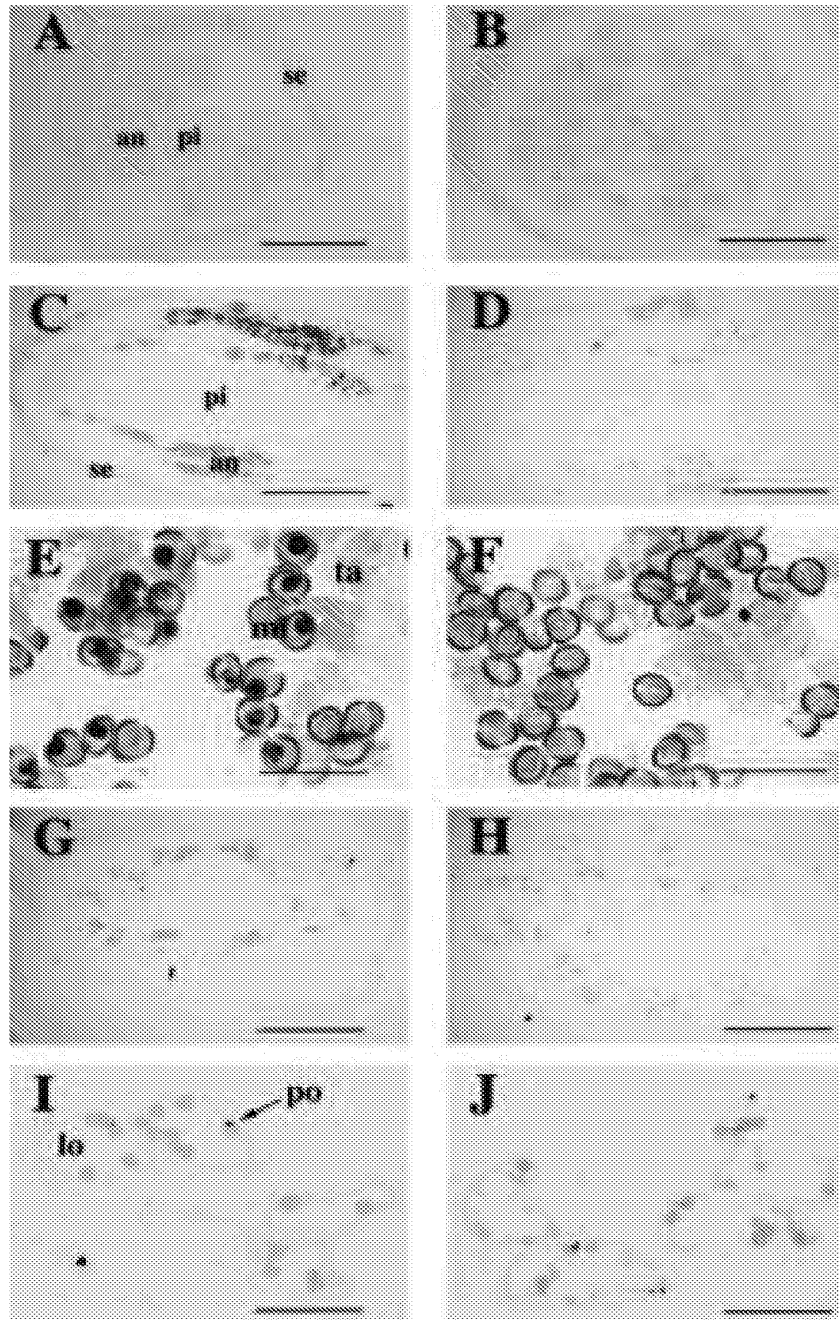


Figure 7. *BnM3.4* expression in floral buds of *B. napus*. *In situ* hybridization of DIG-labelled antisense (A, C, E, G and I) or sense (B, D, F, H and J) *BnM3.4* RNA probe to *B. napus* floral bud sections. A and B. Transverse sections of 1.5 mm long buds containing tetrads (bar = 4 μ m). C and D. Longitudinal sections of 2.5 mm long buds containing microspores (bar = 10 μ m). E and F. Magnification of anthers from transverse sections of 2.5 mm long buds (bar = 0.8 μ m). G and H. Transverse sections of 3.5 mm long buds containing bicellular pollen (bar = 10 μ m). I and J. Transverse sections of anthers from 4.5 mm long buds containing tricellular pollen (bar = 4 μ m). Abbreviations: an, anther; lo, locule; mi, microspore; pi, pistil; po, mature pollen; se, sepal; ta, tapetal cell.

(Figure 7E), the *BnM3* transcripts are clearly visible by a deep staining inside the microspores. No hybridization signal was detected in any male gametophyte tissue at any stage of development with the sense probe (Figure 7B, D, F, H, J). The lack of hybridization of some microspores with the antisense probe is probably an artefact caused by a partial section of the pollen grain or a loss of cytoplasm during the pretreatment of slides. Both the sense and the antisense probes allowed us to detect a weak hybridization signal in tapetal cells that could be considered as a background signal not specific to the *BnM3.4* transcripts.

The BnM3.4 promoter: regions of homology and functional analysis

A detailed analysis of the *BnM3.4* promoter sequence revealed the presence of several regions having possible regulatory functions. Two 14 bp repeats (tt-taaaagtgaac) are located from -1852 to -1780 from the transcription start. A 14 bp palindromic sequence (ttagaatattctaa) is located at -307 while another 12 bp palindrome (ttttaataaaaa) starts at -213. These motifs have not been detected in any other pollen-specific gene. However, the motif 'tcattt', located between -593 and -517 and the reverse motif 'aatga', located at -1566 in *BnM3.4* upstream regions were already described for other pollen-specific genes like *NTP303* (Weterings *et al.*, 1995), *NTM19* (Oldenhof *et al.*, 1996) and *Bp10* (Albani *et al.*, 1992). A 2 kb region of this 5'-flanking sequence, containing these different regions was transcriptionally fused with the reporter *gus* coding sequence. The resulting construction (pJD101, see Materials and methods) was introduced into *A. thaliana*. This species was chosen for these experiments because it belongs to the same botanical family as *B. napus*, but it can be more easily transformed and has a shorter life cycle. GUS staining was first performed on the inflorescences of fifteen independent transgenic *A. thaliana* T₁ lines (JD101 series) transformed with this gene. They all showed qualitatively the same expression patterns in the course of gametophyte development (not shown). The progeny (T₂) of T₁ plants with insertion of the transgene(s)

presumably at a single locus was chosen for further analysis. The results of histochemical analysis of *gus* expression for individual hemizygous and homozygous progeny of one of them, JD101.42, are shown in Figure 8. This transformant presents one insertion according to the genetic test (275 phosphotricine resistant plants out of 380 seedlings; $\chi^2 = 1.40$; $P < 0.05$).

In all the transgenic JD101 plants and their progeny studied, no GUS activity was detected in flower organs other than the stamen as shown in Figure 8A for one hemizygote progeny of the JD101.42 plant. GUS activity was not detected in any organ of seedling (Figure 8I) or older plants (not shown), or in siliques (Figure 8J). In comparison, plants transformed with the control construction pAF100 (*gus* without promoter) showed no GUS activity (Figure 8A, inset). The presence of 50% of blue pollen and 50% of unstained pollen in anthers of the hemizygous JD101.42 plant (Figure 8H) confirms that the *BnM3.4* promoter is only activated in gametophytic cells i.e. only the transgenic pollen shows GUS activity.

The temporal pattern of *gus* expression was determined with homozygous JD101 progeny with the aim of precisely determining the developmental stage where the reporter gene is activated. At the tetrad stage of male gametophyte, no GUS activity was detected (Figure 8B); it only appears at a high level at the beginning of the microspore stage (Figure 8C). As shown in Figure 8E, high levels of GUS activity were detected in pollen. There seems to be a low level of the GUS product in the locule which may be the result of diffusion, but no activity was present in the sporophytic tissues of the anther such as the epidermis, the endothecium and the tapetum. The GUS expression level stays constant until the mature tricellular pollen stage (Figure 8D, 8F and 8G) but it is difficult to know if this is due to the longevity of the GUS protein or transgene activity in *Arabidopsis*.

At least for the first stages of male gametophyte development, the reporter gene has the same expression pattern as intact genes.

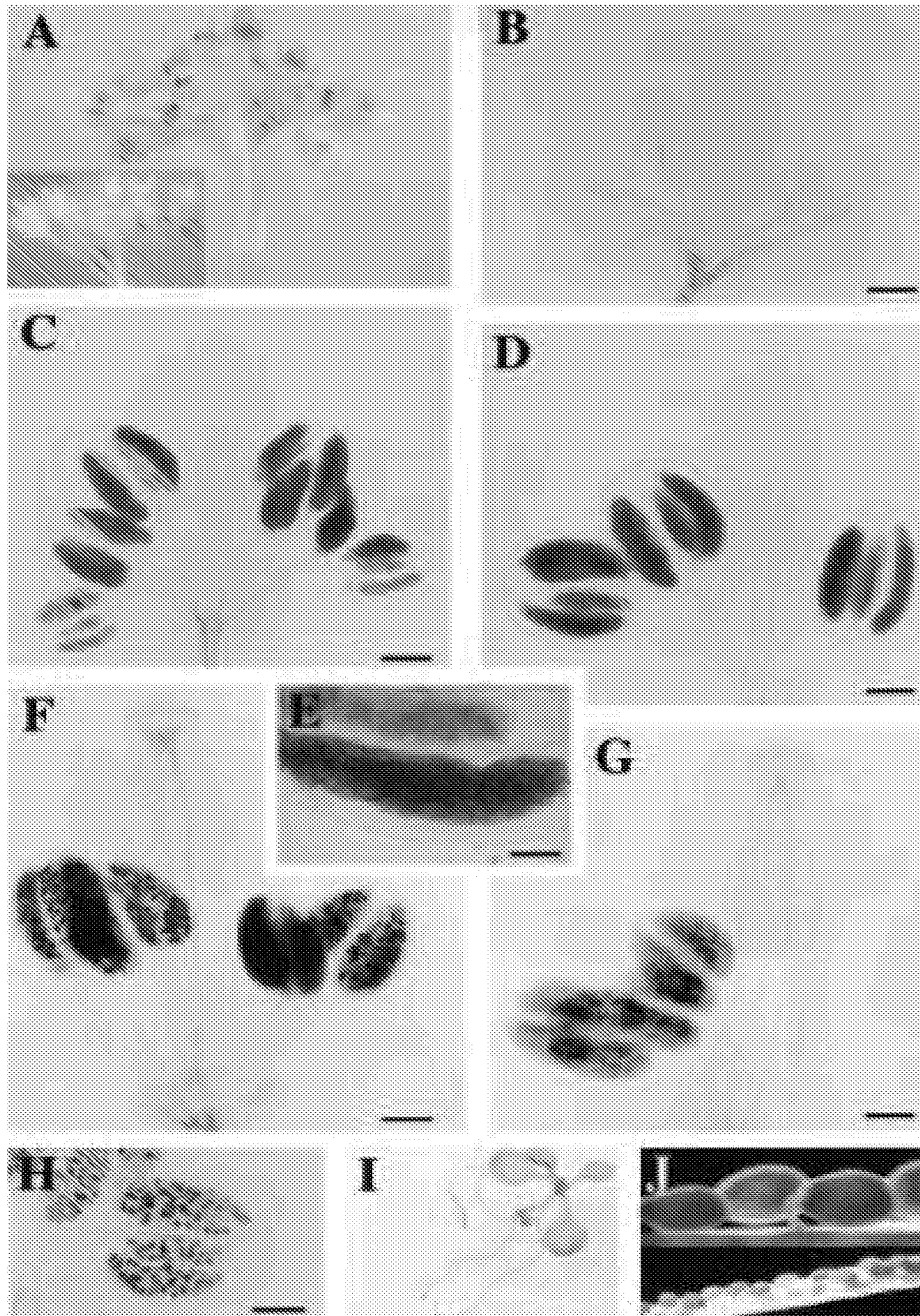


Figure 8. Histochemical analysis of *gus* expression in transgenic *A. thaliana* JD101 plants. The following plant materials from homozygous and hemizygous JD101.42 transgenic plants and from a AF100 transgenic plant were examined for GUS activity as described in Materials and methods. A. Inflorescence from a 5-week old homozygous pJD101 plant and (in the inset) the inflorescence from a 5-week old AF100 plant (negative control plant). B–G. Developmental series of buds from the stained inflorescence presented above. Sepals and petals were removed from the bud in order to measure the pistil length more easily and to improve the visualization of GUS in anthers. The stages of male gametophyte development were determined according to the pistil length as described in Materials and methods. B, tetrad stage; C, early microspore stage; D, end of microspore stage; E, locule of one of the anthers of the bud shown in D, magnified 6× relative to Figure D (bar represents 27 µm); F, bicellular pollen; G, tricellular pollen in anthers at anthesis. In B, C, D, F and G, bars represent 90 µm. H. Anther of a hemizygous plant, at the microspore stage (bar represents 70 µm). I. Ten-day old seedling. J. Open siliques and seeds at two different magnifications.

Discussion

This paper describes a method in order to isolate cDNAs from rapeseed that are preferentially expressed during early male gametophyte development. Expression of several of these genes appears to be highly specific to microspores and we have analysed one of these, called *BnM3.4*, in detail. This gene corresponds to the M3 cDNA isolated, but another cDNA was isolated from the microspore cDNA library using M3 as a probe. This longer cDNA (M3.21) is homologous to M3 except that it shows a deletion of 99 bp in the putative coding region. As these two cDNA cross-hybridize in the hybridization conditions used, we can conclude that at least two genes (the *BnM3* family=*BnM3.4* + related gene(s)) are present in the *B. napus* genome. This is also supported by the results of 5' RACE and Southern blot experiments.

The expression of these genes was studied both by northern blot and *in situ* hybridization. By northern blot analysis, the transcripts were observed mainly in microspores and also in total RNA extracted from buds of length strictly below 2 mm, that contain a majority of tetrads and meiocytes. The developmental synchrony between all the male gametophytes of an anther is not perfect and some free microspores already appear among tetrads in buds below 2 mm, so the hybridization of M3 probe to RNAs from pollen extracted from buds below 2 mm may be due to the early presence of microspores in these buds. This hypothesis was confirmed by *in situ* hybridization experiments. In individual 1.5 mm long buds, where no microspores were found, no signal was detected. On the other hand, in 2.5 mm long buds, where microspores are the only developmental stage present, a strong signal was detected. In later gametogenesis stages, no further signal was detected. According to these results, we can conclude that the homologous genes of *BnM3* family are expressed specifically in the microspore of *B. napus*.

Like the other early-expressed gene isolated from tobacco, *NTM19* (Oldenhof *et al.*, 1996), *BnM3.4* does not show any homology to known genes. Although the deduced *BnM3.4* protein does not show homology with the deduced *NTM19* protein, they are both proline-rich proteins and contain a putative signal peptide. We can only speculate on the function and cellular localisation of the *BnM3.4* protein with the help of prediction programs. Several stamen proteins of unknown function contain similar proline-rich domains (Chen *et al.*, 1993) that, despite differences in amino-acid composition, are structured in a very similar way. The role of the proline-rich motifs is not known, but one may speculate that they serve as some kind of anchoring structures in interaction with either components of the cytoskeleton or with structural components of the cell wall (Woessner *et al.*, 1994; Kröger *et al.*, 1996).

The comparison analysis of the *NTM19* and the *BnM3.4* promoter regions did not show any clear common domains which could indicate a role in the specificity of expression of these genes. Only the box 'tcattt' and its reverse sequence, 'aaatga', which has already been shown to be important for pollen-specific expression are present in both promoters. Even if the number and the precise location of these motifs are not conserved between the different promoter regions described (tobacco for *NTM19* and *NTP303*, rapeseed for *Bp10*), functional analysis using deletion and targeted mutagenesis experiments in microprojectile-mediated transient expression assays (Weterings *et al.*, 1995) showed clearly that one of these elements of the *NTP303* promoter constitutes a positive *cis*-regulatory element and functions by specifically enhancing transcription in pollen. This is the reason why we kept this promoter region of 2 kb for the heterologous expression experiments. Both analysis of more microspore-specific genes and promoter deletions using a reporter gene will be necessary to evaluate the influence of the different motifs in the spacial and temporal regulation of transcription of these genes.

The histochemical analysis of *gus* expression driven by the *BnM3.4* promoter (pJD101 construction) in transgenic *A. thaliana* plants showed a high level of GUS activity in male gametophytes but not in other floral and seedling tissues. Furthermore, GUS activity appeared at the microspore stage and stayed at a constant level during pollen development until pollen maturity. GUS being a very stable protein, the detection of GUS activity allowed us to define precisely the start point of *BnM3.4* promoter activity in *A. thaliana* but not its stop point. The spatial and temporal pattern of *gus* expression driven by the *BnM3.4* promoter in *A. thaliana* is in accordance with the established expression pattern of the gene in *B. napus*. These results suggest the presence of *trans*-acting factors in *A. thaliana* able to recognise and interact with the *cis*-regulatory elements of *BnM3.4* in the same manner as in *B. napus*.

Several of the genes isolated by this procedure belong to gene families (*BnM3.4*, *SKP1*-like genes) as seen with other transcriptional approaches (Twell, 1994). This strategy is complementary to the mutagenesis being undertaken in *A. thaliana* (Feldmann *et al.*, 1997; Bonhomme *et al.*, 1999). We now plan to compare the 5'-flanking regions of the other microspore-specific genes we have isolated and to try *BnM3.4* promoter deletion analysis in order to find *cis*-regulatory elements required for microspore-specific expression.

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Preplant and Preemergence Herbicides for Soybean Production

The following herbicides are used to control weeds as they emerge. Certain products require mechanical incorporation prior to planting, while others may be applied before, during, or after planting. Incorporation may reduce the need for timely rainfall after application and may improve control of certain weeds. Adjust rate to soil type as specified on label. In no-till, an additional burndown herbicide may be required to control emerged weeds. Many herbicides used primarily for pre-emergence weed control may also be applied following crop emergence. Recommendations for post applications of these products are included in this section rather than in the postemergence section.

Trade Name	Common Name	Formulation	Rate/Range
Authority	sulfentrazone	75 DG	4.0 to 5.3 oz/A
Spartan	sulfentrazone	75D DF	5.3 to 8.0 oz/A

Mode of Action

PPO

Application Timing

Early preplant—Apply up to 30 days prior to planting.

Preplant incorporated—Apply and uniformly incorporate no deeper than the top 1 to 2 inches of soil. May be applied up to 30 days prior to planting.

Preemergence—Apply before soybeans emerge.

Comments

- For use in a planned program to be followed by a postemergence application such as Classic, Classic + Roundup Ultra (RR soybean varieties), or Synchrony STS.
- Soybean stunting may occur if excessive rainfall occurs after application and before soybean emergence.
- Do not apply if there are visible signs of cracking due to soybean emergence.
- Thoroughly clean application equipment after use to avoid crop injury. Refer to label for cleaning instructions.
- Do not apply aerially.
- Do not apply through any type of irrigation system.
- Some soybean varieties demonstrate less tolerance to sulfentrazone. Refer to the label for a list of these varieties.

Trade Name	Common Name	Rate/Range
Axiom 68 DF	flufenacet 54.4% + metribuzin 13.6%	7 to 13 oz/A

Mode of Action

Unknown + PSII

Application Timing

Preplant surface—Applications may be made up to 14 days before planting.

Preplant incorporated—Apply and incorporate into the top 1 to 2 inches of soil up to 14 days before planting.

Preemergence—Apply after planting but prior to soybean or weed emergence.

Comments—Axiom 68DF

- Axiom cannot be mixed or loaded within 50 feet of any well, lake, river, stream, reservoir, or sinkhole. This does not apply to properly capped or plugged wells, impervious pads, or properly diked mixing and loading areas.
- Do not apply aerially or with irrigation systems.
- Soybeans should be planted a minimum of 1 to 1½ inches deep.
- Axiom is labeled at lower rates in soybeans than corn due to residue limits rather than to lower tolerance. The labeled rates will only provide early season weed control.

Trade Name	Common Name	Rate/Range
Boundary 7.8EC	6.3 lb S-metolachlor + 1.5 lb metribuzin	1 to 2.5 pt/A

Mode of Action

Unknown + PSII

Application Timing

Preplant surface or incorporated—Apply up to 14 days before planting. If incorporating, mix into the upper 1 to 2 inches of soil.

Preemergence—Apply after planting but prior to soybean or weed emergence.

Comments

- Intended for use with a planned postemergence program. Boundary will provide 4 to 6 weeks residual control.
- On soils with pH above 7.0, use the 1.25 pt/A rate only.
- 1.25 pt/A Boundary provides the equivalent of 1 pt/A Dual II MAGNUM plus 4.9 oz/A Sencor 75DF.
- Refer to label for other comments relative to postemergence products following Boundary and burndown of existing vegetation at the time of planting.
- Do not use on sand soils. On coarse textured soils, do not use on loamy sands with less than 2% organic matter.

Trade Name	Common Name	Rate/Range
Canopy XL 56.3 DF	46.9% sulfentrazone + 9.4% chlorimuron ethyl	2.5 to 7.9 oz/A

Mode of Action

PPO + ALS

Application Timing

Fall applications—Apply 2.5 oz after fall harvest but before ground freeze to control winter annual weeds.

Early preplant—Apply up to 30 days prior to planting. Will provide some burndown of existing weeds.

Preplant incorporated—Apply and uniformly incorporate no deeper than 1 to 2 inches deep in the soil. May be applied up to 30 days prior to planting.

Preemergence—Apply before soybeans emerge.

Comments

- Do not apply on fields with a pH greater than 6.8.
- Chlorimuron has a long soil residual and may carry over and injure corn. Carryover of chlorimuron is greater on soils with a high pH. Refer to label for rotational restrictions. Chlorimuron may interact with ALS herbicides applied to corn the following year, thus increasing the potential for injury to corn.
- Consider potential for localized areas in field that may have a high pH resulting in carryover. Composite soil samples taken for fertility testing may not reveal these areas.
- Do not plant field corn for 10 months after application or Clearfield field corn for 8 months.
- Soybean injury may occur if excessive rainfall occurs after application.

Comments—Canopy, continued

- Canopy XL may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Fall applications intended for controlling winter annuals. Will provide limited activity on spring-germinating weeds.
- Apply in a minimum of 10 gallons of water per acre.
- Do not apply aerially.
- Thoroughly clean sprayer following use to avoid crop injury. Refer to label for cleaning instructions.
- Do not graze treated fields or harvest for forage or hay.
- When used for burndown, add 1% v/v crop oil concentrate. Assure II or Roundup Ultra may be included to improve grass control. Substitute 0.5% NIS for the COC when mixing with Roundup Ultra.

Trade Name	Common Name	Formulation	Rate/Range
Command 4EC	clomazone	4 EC	1 to 2 pt/A
Command 3ME		3ME	1½ to 2½ pt/A

Mode of Action

Bleacher

Application Timing—Command 4EC

Early preplant—Apply Command 4EC prior to field green-up (trees or shrubs bordering the treatment site have broken dormancy and are showing green leaf tissue) but no later than April 1 south of I-80 and April 10 north of I-80.

Preplant incorporated—Apply Command 4EC and incorporate immediately unless the soil is dry. On dry soils, incorporation must be completed within 8 hours of application. Shallow incorporation generally results in better weed control than deep incorporation.

Preemergence—Apply Command 3ME before soybeans emerge. May be applied up to 30 days before planting.

Comments

- Off-site movement of spray drift or vapors of Command can cause foliar whitening or yellowing of nontarget plants. Prior to making applications, read and strictly follow all precautions and instructions on the label.
- Command 3ME is an encapsulated formulation. Encapsulation reduces volatilization from the soil surface, thereby reducing the likelihood of off-target movement and injury to susceptible plants in adjacent areas.
- Do not apply within 1,200 ft of towns, subdivisions, commercial fruit and vegetable production, nurseries, or greenhouses. Refer to label for other restrictions.
- Command may carry over and cause early season injury to rotational crops.
- Do not plant wheat, oats, barley, rye, or alfalfa in the fall of the year of application or in the spring of the following year.
- Thoroughly clean sprayer following use to avoid crop injury. Refer to label for cleaning instructions.
- May be applied with fluid fertilizer; do not apply aerially.
- Do not allow livestock to graze on treated soybean vines or harvest vines for forage.

Trade Name	Common Name	Rate/Range
Command Xtra	39.6% sulfentrazone + 31.4% clomazone	8 to 12 oz/A + 21.3 to 32 oz/A

Mode of Action

PPO + bleacher

Application Timing

Early preplant—Apply up to 30 days prior to planting. May provide some burndown of existing weeds.

Preemergence—Apply before soybeans emerge.

Comments—Command

- Do not apply on fields with soils classified as sand with less than 1% organic matter.
- Do not apply more than once per season.
- Do not allow livestock to graze on treated soybean vines, or feed treated soybean vines or vine trash to livestock.
- Command Xtra is a co-pack containing 3 quarts Command Xtra B and 2 gallons Command Xtra G.
- Each Command Xtra co-pack will treat 8 to 12 acres depending on the soil type.

Trade Name	Common Name	Rate/Range
Domain 60DF	24% flufenacet + 36% metribuzin	9 to 16 oz/A

Mode of Action

unknown + PSII

Application Timing

Preplant surface or incorporated—Apply up to 14 days prior to planting. If incorporating, mix into upper 1 to 2 inches of soil.

Preemergence—Apply following planting but before crop or weed emergence.

Comments

- Intended for use with planned postemergence program. Domain will provide only early season (from 3 to 6 weeks) residual control.
- Crop injury may occur when 1) soils have a calcareous surface area or a pH of 7.5 or greater, 2) a soil-applied OP insecticide is used, or 3) the product is applied or incorporated unevenly.
- Sixteen oz/A Domain provides the equivalent of 0.5 lb Sencor 75DF/A.
- Flufenacet is similar in activity to metolachlor, acetochlor, and other amide herbicides.

Trade Name	Common Name	Formulation	Rate/Range
Dual II MAGNUM	s-metolachlor	7.64 EC	1.0 to 2.0 pt/A
Dual II MAGNUM G	s-metolachlor	16G	6 to 15 lb/A
Parallel	metolachlor	7.8 EC	1.0 to 2.0 pt/A
Parallel PCS	metolachlor	8 EC	1.0 to 2.75 pt/A
Stalwart	metolachlor	8 EC	1.0 to 2.0 pt/A

Mode of Action

Unknown

Application Timing

Fall application—Apply 1.67 to 2.0 pt/A of s-metolachlor or metolachlor after September 30 north of U.S. 30 or after October 15 south of U.S. 30 when soil temperature at a 4-inch depth is less than 50°F. Do not apply to frozen soil.

Early preplant—Apply up to 30 days ahead of planting as a single application or up to 45 days ahead of planting as a split application.

Preplant incorporated—Apply and incorporate into the top 2 inches within 14 days before planting.

Preemergence—Apply during or after planting but before weeds or crops emerge.

Comments—Dual II MAGNUM

- The higher rates are required on soils with high organic matter or for control of black nightshade.
- May be applied with liquid or dry fertilizers, through center pivot irrigation, or aerially.
- Treated areas may be grazed or used for forage.
- Iowa State University research has found that fall applications increase the likelihood of late-season weed breakthroughs due to early degradation of s-metolachlor and metolachlor.
- Parallel, Parallel PCS, and Stalwart are formulated with the unresolved isomers of metolachlor and will have less of the isomer primarily responsible for weed control as found in s-metolachlor.

Trade Name	Common Name	Formulation	Rate range
Express	tribenuron	75% DF	1/6 to 1/3 oz

Mode of Action

ALS

Application Timing

Fall application — Apply in the fall after harvest but before ground freeze.

Comments

- Do not plant corn or soybean within 45 days of application.
- Express will provide control of chickweed and certain other winter annuals.

Trade Name	Common Name	Formulation	Rate/Range
FirstRate/Amplify	cloransulam-methyl	84% WDS	0.6 to 0.75 oz/A

Mode of Action

ALS

Application Timing**Preplant incorporated**—Apply and incorporate into the top 1 to 3 inches of soil up to 4 weeks prior to planting.**Preplant surface**—Apply up to 4 weeks prior to planting.**Preemergence**—Apply after planting but before crop emergence.**Comments**

- FirstRate can be applied postemergence. Refer to the label to determine the proper application rate.
- A post-application of 0.3 oz/A FirstRate may follow a soil application of 0.75 oz/A or less.
- Do not apply more than 1.05 oz/A FirstRate per growing season.
- FirstRate may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Giant ragweed populations resistant to FirstRate have been identified in Iowa and some adjacent states.
- Refer to the FirstRate label for specific rotational information.
- Cloransulam occasionally has injured corn the year following application. Late applications, below average precipitation after application, and interactions with other ALS herbicides increase the potential for adverse crop response.

Trade Name	Common Name	Formulation	Rate/Range
Frontier	dimethenamid	6.0 EC	16 to 32 oz/A
Outlook	dimethenamid-P	6.0 EC	12 to 21 oz/A

Mode of Action

Unknown

Application Timing

Early preplant—Use split applications made 30 to 45 days before planting. Treatments made 30 days or less before planting may be made as a single application. Use the highest rate recommended for the soil type.

Preplant incorporated—Incorporate 1 to 2 inches deep up to two weeks before planting.

Preemergence—Apply after planting and before crop emergence.

Early postemergence—May be applied up to the third trifoliolate stage.

Comments

- Do not graze or feed forage, hay, or straw to livestock.
- If conditions of high rainfall and water-saturated soil occur during soybean germination or seedling development, Frontier may suppress soybean development.
- Frontier may be applied at reduced rates of 15 to 20 oz/A when used in postemergence tank mixes with Pursuit.
- Temporary soybean burn and stunting may occur if applied to emerged soybeans.
- Outlook contains the resolved isomer of dimethenamid.

Trade Name	Common Name	Formulation	Rate/Range
Gangster	51% flumioxazin +	84% cloransulam-methyl	1.5 to 3 oz/A + 0.3 to 0.6 oz/A

Mode of Action

PPO + ALS

Application Timing

Early preplant—Apply up to 30 days prior to planting.

Preemergence—Apply before soybeans emerge.

Comments

- Do not apply Gangster to soils classified as sandy that contain less than 1% organic matter.
- If applied after soybean emergence, soybean injury will occur.
- Do not apply Gangster when weather conditions favor spray drift.
- Do not graze treated fields or feed forage or hay to livestock.

Trade Name	Common Name	Rate/Range
Gauntlet	75% sulfentrazone + 84% cloransulam-methyl	5.33 to 6.67 oz/A + 0.6 to 0.75 oz/A

Mode of Action

PPO + ALS

Application Timing—Gauntlet

Early preplant—Apply up to 30 days prior to planting. May provide some burndown of existing weeds.

Preplant incorporated—Apply up to 30 days prior to planting and incorporate in the top 1 to 3 inches of soil.

Preemergence—Apply before soybeans emerge.

Comments

- Do not apply if soybeans have begun to emerge, as injury may result.
- Do not apply by air or through any type of irrigation system.
- Do not make more than one application per season.
- Do not feed treated soybean forage or soybean hay to livestock.
- Do not harvest soybeans for 65 days after Gauntlet application.
- Do not apply to soils classified as sands containing less than 1% organic matter.
- Gauntlet is a co-pack containing 26.5 oz sulfentrazone and five water-soluble packets, each containing 0.6 oz FirstRate (8:1).
- Cloransulam occasionally has injured corn the year following application. Late applications, below average precipitation after application, and interactions with other ALS herbicides increase the potential for adverse crop response.

Trade Name	Common Name	Formulation	Rate/Range
Lasso EC, MicroTech	alachlor	4 EC, ME	2 to 3.0 qt/A
Intro		4 EC	2 to 3.0 qt/A

Mode of Action

Unknown

Application Timing

Early preplant—MicroTech may be applied up to 30 days ahead of planting as a single application, or up to 45 days ahead of planting as a split application.

Preplant incorporated—Apply within 7 days of planting and incorporate into the top 2 inches of soil.

Preemergence—Apply after planting, before weed and crop emergence, and within 5 days of last preplant tillage operation.

Comments

- All products containing alachlor are restricted-use pesticides and contain a groundwater protection statement.
- MicroTech and Partner are encapsulated formulations of alachlor.
- The higher rates are required on soils with high organic matter or for control of black nightshade.
- Lasso and MicroTech may be applied in fluid fertilizer.
- Do not apply by air; do not graze treated area or feed treated forage to livestock.
- Refer to specific alachlor label for tank mixing and application instructions.

Trade Name	Common Name	Formulation	Rate/Range
Lorox DF	linuron	50 DF	1 to 5 lb/A
Lorox		4 L	1 to 5 pt/A

Mode of Action

PSII

Application Timing

Preemergence—Apply after planting but before crop emerges.

Comments—Lorox

- Soybeans should be planted at least 1¾ inches deep to reduce risk of injury. Injury from Lorox appears as yellowing (chlorosis) followed by necrosis of lower leaves.
- Heavy rainfall following application on a poorly prepared seedbed may result in severe injury.
- Lorox is not effective on many of the high organic matter soils found in the Clarion-Nicollet-Webster soil association.
- Do not apply in liquid fertilizer; may be applied aerially.

Trade Name	Common Name	Formulation	Rate/Range
Prowl	pendimethalin	3.3 EC	1.2 to 3.6 pt/A
Pendimax		3.3 EC	1.2 to 3.6 pt/A
Pentagon		60 DG	0.85 to 2.5 lb/A
Prowl H ₂ O		3.8 ACS	1.5 to 3.0 pt/A

Mode of Action

Microtubule

Application Timing

Preplant incorporated—Incorporate 1.2 to 3.6 pt EC or equivalent/A into the soil within 7 days after application, and plant soybeans within 60 days after application. Prowl H₂O may be applied up to 60 days prior to planting and incorporated.

Preplant surface—May be surface applied up to 45 days before planting. Prowl at 1.2 to 3.6 pt/A applied alone should be followed by a postemergence application of Pursuit.

Preemergence—Apply 1.2 to 3.0 pt/A at planting or up to 2 days after planting.

Fall application—May be applied following harvest but before soil freeze-up.

Comments

- Do not apply preemergence north of I-80 due to increased potential for crop injury.
- Prowl H₂O is a water-based encapsulated formulation. Encapsulation may reduce volatilization losses associated with surface applications and allow easier cleaning of equipment after spraying compared to the EC formulation.
- At label rates, Prowl may occasionally carry over and injure rotational crops. Carryover is most likely in areas of overlaps or other areas of misapplication.
- May be applied in the fall when followed with an early postemergence application of Pursuit. Iowa State University does not recommend fall applications.

Trade Name	Common Name	Rate/Range
Pursuit Plus	2.7 lb pendimethalin + 0.2 lb imazethapyr/gal	2.5 pt/A

Mode of Action

Microtubule + ALS

Application Timing

Preplant surface applications—May be surface applied up to 45 days prior to soybean planting. Mechanical incorporation is recommended if sufficient rainfall to activate herbicide does not occur prior to planting.

Preplant incorporated—Apply following land preparation and thoroughly incorporate to a depth of 1 to 2 inches within 7 days of application.

Preemergence—May be surface applied up to 2 days after soybean planting south of I-80 only. Do not apply after soybean planting north of I-80.

Comments—Pursuit Plus

- The components of Pursuit Plus have long soil residual characteristics and may carry over. Refer to label for specific rotational restrictions.
- Mechanically incorporated treatments have provided more consistent control than surface-applied treatments.
- Do not apply aerially. Do not graze or feed treated forage. Do not apply products containing chlorimuron ethyl, imazaquin, or imazethapyr the same year as Pursuit Plus.
- Pursuit Plus may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- See remarks under Pursuit and Prowl.
- Imazethapyr may interact with ALS herbicides applied to corn the following year, thus increasing the potential for injury to corn.

Trade Name	Common Name	Formulation	Rate/Range
Python	flumetsulam	80 WDG	0.8 to 1.33 oz/A

Mode of Action

ALS

Application Timing

Preplant incorporated and preemergence—Apply from 30 days prior to planting until before the cracking stage.

Comments

- Do not harvest within 85 days of application.
- Do not use on soils with pH greater than 7.8.
- Do not use on soils with more than 5% organic matter if the soil pH is less than 5.9.
- Do not use on peat or muck soils.
- Do not graze or feed treated soybean forage, hay, or straw to livestock.
- Python may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Do not apply aerially.

Trade Name	Common Name	Formulation	Rate/Range
Sencor	metribuzin	75 DF	1/3 to 2/3 lb/A
Sencor		4 F	3/4 to 1 1/4 pt/A

Mode of Action

PSII

Application Timing

Preplant incorporated—Metribuzin may be applied between 7 and 21 days ahead of planting, depending upon tank mix partner. Refer to label for specific restrictions.

Preemergence—Apply at or after planting but prior to soybean emergence.

Comments

- Injury potential is greater on soils with a pH greater than 7. Do not use on soils with a pH greater than 7.5.
- Injury appears as yellowing or bronzing of lower leaves, followed by necrosis and sometimes stand reductions.
- Plant seed at least 1 1/2 inches deep to reduce risk of crop injury.
- Treated vines may be grazed or fed to livestock 40 days after application.

Trade Name	Common Name	Formulation	Rate/Range
Sonalan	ethalfluralin	3 HFP	1.5 to 3.5 pt/A

Mode of Action

Microtubule

Application Timing

Preplant incorporated—Incorporate into the top 2 to 3 inches of soil within 2 days after application. May be applied up to 3 weeks prior to planting.

Comments

- Sonalan is similar to Treflan in performance but has reduced risk of carryover. Soybeans have slightly less tolerance to Sonalan than to Treflan.
- Will provide partial control of black nightshade when using higher rates (up to 3½ pt/A) and two-pass incorporation. Higher rates increase risk of crop injury.
- Do not graze or forage soybean vines treated with Sonalan.
- Do not use Sencor combinations on coarse-textured soils with less than 1% organic matter.
- Refer to all labels when applying tank mix combinations.

Trade Name	Common Name	Rate/Range
Squadron	2.0 lb pendimethalin + 0.33 lb imazaquin/gal	3.0 pt/A

Mode of Action

Microtubule + ALS

Application Timing

Preplant incorporated—Incorporate into the top 1 to 2 inches of soil immediately before or up to 45 days before planting. Incorporate within 7 days of application.

Preemergence—Apply up to 45 days before, during, or after planting but prior to soybean emergence.

Comments

- Do not apply postemergence to soybeans.
- Do not plant corn the year following Squadron application.
- Incorporate Squadron + Prowl combinations north of I-80.
- Do not apply through chemigation systems.
- Do not graze or feed treated soybean forage, hay, or straw to livestock.
- Apply Squadron only once during the growing season.
- Iowa State University does not recommend the use of 2,4-D amine formulations.
- Iowa State University does not recommend the use of 2,4-DB.
- Squadron may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Imazaquin has long soil residual characteristics and has demonstrated carryover to rotational corn.
- Refer to Squadron label for specific rotational recommendations.
- Do not use products containing chlorimuron ethyl, flumetsulam, imazethapyr, or imazaquin the same year as Scepter application, unless labeled otherwise, due to carryover to rotational corn.
- There is a 90-day interval between Squadron application and soybean harvest.

Trade Name	Common Name	Formulation	Rate/Range
Treflan HFP	trifluralin	4 EC	1 to 2 pt/A
Treflan TR-10/Trust 10G		10% granule	5 to 10 lb/A
Trifluralin, Trust 4EC, generic brands		4 EC	1 to 2 pt/A

Mode of Action

Microtubule

Application Timing

Preplant incorporated—Treflan must be incorporated one time within 24 hours after application; a second pass should be made any time prior to planting. Incorporate into the top 2 to 3 inches of soil.

Comments

- May be applied aerially or with liquid or dry fertilizer.
- At labeled rates, trifluralin may occasionally carry over and injure rotational crops. Carryover is most likely in areas of overlaps or other areas of misapplication.
- There are no limitations on crop use following trifluralin application.
- Treflan may be applied in the fall from October 15 to December 31; however, ISU does not recommend this application.
- Refer to all labels when applying a tank mix.

Postemergence Herbicides for Soybean Production

The following herbicides are used to control established weeds following soybean emergence. Some products are labeled for both preemergence and postemergence applications.

Trade Name	Common Name	Formulation	Rate/Range
Aim/Avalanche	carfentazone-ethyl	2EW	0.25 – 1.6 oz/A

Mode of Action

PPO

Application Timing

Apply up to 30 days before planting until soybeans reach the third trifoliolate. Weeds should be emerged and actively growing but no more than 4 inches tall or rosettes are less than 3 inches in diameter. Apply Aim at 0.25 oz/A broadcast for velvetleaf control. Aim/Avalanche may be applied at 0.5 to 1.6 oz/A as a post-directed treatment.

Comments

- Aim/Avalanche can be applied broadcast only at a rate of 0.25 oz/A. Apply higher rates as a post-directed application.
- Do not apply when wind speed is greater than 10 MPH.
- Do not tank mix with EC formulations or use crop oil concentrate.
- If spray contacts soybean foliage, severe injury can occur.

Trade Name	Common Name	Formulation	Rate/Range
Assure II	quizalofop-p-ethyl	0.8 EC	5 to 10 oz/A

Mode of Action

ACCase

Application Timing

Apply to young, actively growing grasses with size range listed on label. Apply prior to 80 days before harvest. Do not apply after soybean pod set.

Recommended Spray Additives

Crop oil concentrate-1% v/v; or nonionic surfactant-0.125-0.25% v/v.

Comments

- Refer to the label for specific additive recommendations.
- Use lower rate of surfactant with Pinnacle tank mixes.
- Tank mixing with broadleaf herbicides may reduce grass control. Rates may be increased by 2 oz when used in tank mixes to overcome antagonism for some grass species.
- May be applied aerially.
- Do not graze treated fields or harvest for foliage or hay.
- Do not tank mix Assure II with other herbicides when treating yellow foxtail, woolly cupgrass, barnyardgrass, crabgrass, quackgrass, or wirestem muhly.
- When making a sequential application, apply Assure II and delay application of broadleaf herbicide 24 hours, or apply the broadleaf herbicide and delay Assure II application 7 days.
- Do not use vegetable-based oils as adjuvants with Assure II.
- Do not cultivate within 7 days prior to or after Assure II application.
- Assure II is rainfast within 1 hour of application.
- Do not apply more than 18 oz/A Assure II per season.
- Do not apply Assure II within 80 days of harvest or after pod set has begun.
- May be applied early preplant as a burndown tank-mix with Authority, Canopy XL and/or 2,4-D. Refer to the specific labels for restrictions.

Trade Name	Common Name	Formulation	Rate/Range
Basagran	bentazon	4 L	1 to 2 pt/A

Mode of Action

PSII

Application Timing

Apply when weeds are small and actively growing and before weeds reach maximum size listed on label.

Recommended Spray Additives

Crop oil concentrate-1 to 2 pt/A; UAN-0.5 to 1 gal/A; or ammonium sulfate-2.5 lb/A or 3 qt/A.

Comments–Basagran

- Additive selection should be based on weed species present. Do not use both COC and UAN.
- Thorough coverage of foliage required for adequate control. Apply in a minimum of 20 gal of water/A and a minimum of 40 PSI.
- Do not cultivate within 5 days before or after application.

Trade Name	Common Name	Formulation	Rate/Range
Blazer, Ultra Blazer	acifluorfen	2 L	0.5 to 1.5 pt/A

Mode of Action

PPO

Application Timing

Apply early when weeds are small and actively growing and before weeds reach maximum size listed on label. Do not apply within 50 days of harvest.

Recommended Spray Additives

Nonionic surfactant-1 pt per 100 gal water; higher rates may be used for specific weeds. UAN-0.5 to 1 gal/A. Crop oil concentrate-1 to 2 pts/A.

Comments

- Soybean leaves may exhibit burning, crinkling, and bronzing, particularly on young foliage. Injury is normally short lived.
- Do not cultivate within 5 days before or 3 to 7 days after application.
- Thorough coverage of foliage required for adequate control. Use a minimum of 20 gal water and 40 PSI.
- Additive selection based upon target species and tank mix partner.
- Do not use flood, whirl chamber, or CDA nozzles.
- Do not use treated plants for feed or forage.

Trade Name	Common Name	Formulation	Rate/Range
Classic	chlorimuron ethyl	25 DF	0.25 to 0.75 oz/A

Mode of Action

ALS

Application Timing

Should be applied to young, actively growing weeds. Can be applied after first trifoliolate until 60 days before harvest.

Recommended Spray Additives

Nonionic surfactant-0.25% v/v; or crop oil concentrate-1% v/v. UAN (2-4 qt/A), 10-34-0 (1-2 qt/A) or ammonium sulfate (2-4 lb/A) should be used in combination with a surfactant or COC for velvetleaf control. Use only 0.5% (v/v) COC with Cobra tank mixes and NIS with Roundup Ultra tank mixes.

Comments

- Apply in a minimum of 10 gal water. Use flat fan or hollow cone nozzles.
- Cultivation before, during, or within 7 days after application is not recommended.
- Follow label directions for cleaning sprayer after use.
- Do not graze treated fields or harvest for forage or hay.
- Classic may be aerially applied.

Comments—Classic, continued

- Classic may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Classic may interact with ALS herbicides applied to corn the following year, thus increasing the potential for injury to corn.
- Do not apply to plants under stress.
- There is a 60-day interval between application and harvest.
- High soil pH (greater than 7.0) increases the persistence of Classic. Refer to the Classic label for specific restrictions.
- Do not use Dash adjuvant with Classic + Pinnacle combinations.
- ISU does not recommend 2,4-DB.
- Apply Classic + Roundup Ultra on RR soybean varieties only.

Trade Name	Common Name	Formulation	Rate/Range
Cobra	lactofen	2 EC	6 to 12.5 oz/A
Phoenix	lactofen	2 EC	8 to 12.5 oz/A

Mode of Action

PPO

Application Timing

Apply to small, actively growing weeds that are not larger than listed on label.

Recommended Spray Additives

Cobra -COC-Use 0.25 to 1% (v/v); or NIS-Use 0.25% (v/v) when soil moisture is good and weeds are actively growing; or AMS-4 lb when weeds are actively growing. Additive recommendations are based upon weed size and environmental conditions. See label for specific recommendations.

Phoenix – Add NIS at 0.125 to 0.25% v/v COC is recommended when weeds are stressed due to hot and dry conditions.

Under these conditions, use COC at 1 pt/A or use COC at 0.125 to 0.5% v/v plus NIS at 0.125 to 0.25% v/v. When Phoenix is applied in combination with glyphosate, use AMS at 2 to 4 lb/A only.

Comments

- Soybean injury is likely, although plants usually recover with no impact on yield potential.
- Apply in 20 to 30 gal water at 40-60 PSI. Do not use flood nozzles.
- Do not graze or harvest for feed or bedding.
- Do not apply after growth stage R6 (full seed) or within 45 days of harvest.
- Multiple applications may be made. Do not exceed 25 oz/A/season.
- Do not make band applications while cultivating or create excessive dust while spraying.

Trade Name	Common Name	Formulation	Rate/Range
Extreme	1.8% imazethapyr 22% glyphosate	2.17 E	3 pt/A

Mode of Action

ALS + EPSPS

Application Timing

Apply to actively growing weeds within the recommended stage of growth on label.

Recommended Spray Additives

Use a non-ionic surfactant (0.125% v/v) and fertilizer such as a spray grade ammonium sulfate (2.5 lb/A) or a nitrogen-based liquid fertilizer such as 28% UAN (1.1 to 2 qt/A).

Comments—Extreme

- 2,4-D may be added to improve perennial weed control when Extreme is applied as a burndown in no-tillage systems.
- Do not apply postemergence to non-glyphosate-resistant soybeans.
- Do not graze or feed treated soybean forage, hay, or straw to livestock.

Trade Name	Common Name	Formulation	Rate/Range
FirstRate/Amplify	cloransulam-methyl	84% WDS	0.3 oz/A

Mode of Action

ALS

Application Timing

Apply from soybean emergence until soybeans reach 50% flowering.

Recommended Spray Additives

Nonionic surfactant (0.125 to 0.25%) plus UAN (2.5%), or crop oil concentrate or methylated seed oil (1.2%), or crop oil concentrate or methylated seed oil (1.2%) plus UAN (2.5%).

Comments

- FirstRate can be applied preplant incorporated and preemergence. Refer to the label to determine the proper application rate.
- A post application of 0.3 oz/A FirstRate may follow a soil application of 0.75 oz/A or less.
- Do not apply more than 1.05 oz/A FirstRate per growing season.
- FirstRate may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Application prior to full emergence of the first soybean trifoliolate may cause temporary chlorosis.
- Giant ragweed populations resistant to FirstRate have been identified in Iowa and some adjacent states.
- Refer to the FirstRate label for specific rotational information.
- Cloransulam occasionally has injured corn the year following application. Late applications, below average precipitation after application, and interactions with other ALS herbicides increase the potential for adverse crop response.

Trade Name	Common Name	Formulation	Rate/Range
Flexstar	fomesafen	1.88 ME	1 to 1.25 pt/A
Reflex	fomesafen	2 LC	0.75 to 1.25 pt/A

Mode of Action

PPO

Application Timing

Apply to actively growing weeds within recommended growth stages. Apply before soybeans bloom.

Recommended Spray Additives**Flexstar**

MSO or COC-0.5 to 1.0% v/v. Include a minimum of 2.5% v/v UAN or a minimum of 10 lb AMS/100 gal water.

Reflex

Nonionic surfactant-0.25% v/v; or crop oil concentrate-0.5% to 1% v/v. UAN (1 gal/A), DAP (2 pt/A), or AMS may be used in addition to the NIS or COC.

Comments–Flextar, Reflex

- Do not apply fomesafen to any field more than once every two years. Refer to label for rotational restrictions.
- Apply in a minimum of 15 gal water with 30-60 PSI. Do not use flood nozzles.
- Do not graze treated areas or harvest for hay or forage.
- May be aerially applied.

Trade Name	Common Name	Formulation	Rate/Range
Fusilade DX	fluazifop-p-butyl	2 EC	6 to 12 oz/A

Mode of Action

ACCase

Application Timing

Apply to actively growing grasses at recommended growth stages on label. Apply to soybeans prior to bloom.

Recommended Spray Additives

Crop oil concentrate-0.5 to 1% v/v; or nonionic surfactant-0.25 to 0.5% v/v. UAN (1 gal/A) or ammonium sulfate may be used in combination with either the COC or surfactant.

Comments

- Tank mixing may reduce grass control. Refer to label for directions regarding tank mixes.
- Apply in 5 to 40 gal water with pressure of 40-60 PSI. Do not use flood tips. May be applied aerially.
- Cultivation is not recommended within 7 days prior to or 7 days after application.
- Do not graze or harvest for forage or hay.

Trade Name	Common Name	Rate/Range
Fusion 2.66 EC	2.0 lb fluazifop-p-butyl + 0.66 lb fenoxaprop-ethyl/gal	6 to 14 oz/A

Mode of Action

ACCase + ACCase

Application Timing–Fusion 2.66 EC

Apply to actively growing grasses within the stage of growth recommended on the label. Apply prior to soybean bloom.

Recommended Spray Additives

Crop oil concentrate-0.5 to 1% v/v; or nonionic surfactant-0.25 to 0.5% v/v. In addition to COC or NIS, UAN or ammonium sulfate may be added to the spray mixture. The fertilizer additives should not be a substitute for the COC or NIS.

Comments

- Tank mixtures with broadleaf herbicides may reduce grass control. Refer to the Fusion label for tank mix and sequential recommendations.
- Apply in 5 to 40 gal water/A with 40-60 PSI. Do not use flood tips.
- May be aerially applied.
- Cultivation is not recommended 7 days prior to or 7 days after application.
- Do not graze or harvest soybeans for forage or hay.
- May be tank mixed with 2,4-D (LVE) and applied preplant. Refer to label for directions.

Trade Name	Common Name	Formulation	Rate/Range
Harmony GT	thifensulfuron methyl	75 DF	1/12 oz/A

Mode of Action

ALS

Application Timing

Apply to weeds that are young but before they exceed size indicated on label. May be applied after first trifoliolate has fully expanded but no later than 60 days before harvest.

Recommended Spray Additives

Nonionic surfactant-0.125 to 0.25% v/v; or crop oil concentrate-0.5% v/v. UAN (2 to 4 qt/A), 10-34-0 (1-2 qt/A) or ammonium sulfate (2-4 lb/A) in addition to a surfactant is required for control of velvetleaf.

Comments

- Harmony GT is a concentrated formulation of thifensulfuron, previously marketed as Pinnacle.
- May cause soybean injury under hot and humid weather conditions. Use of crop oil concentrate increases injury potential. Injury appears as yellowing of leaves, shortened internodes, red veins, and occasionally death of apical growing point.
- Do not tank mix with Poast Plus.
- Do not make more than one application of Harmony per growing season.
- Apply no later than 60 days before harvest.
- Apply after the first trifoliolate has fully expanded.
- Do not use Dash unless specified on other DuPont supplemental labeling.
- Harmony may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Do not cultivate before, during, or within 7 days after Harmony application.
- Do not graze or feed forage, hay, or straw from treated areas to livestock.
- Do not apply through chemigation systems.
- Do not tank mix Harmony with any other herbicide unless specifically labeled due to potential for excessive crop injury.

Trade Name	Common Name	Formulation	Rate/Range
Poast Plus	sethoxydim	1 EC	18 to 36 oz/A

Mode of Action

ACCase

Application Timing

Apply to actively growing grasses within the recommended stage of growth on label.

Recommended Spray Additives

Crop oil concentrate-2 pt/A; or Dash HC-1 pt/A. May include AMS (2.5 lb/A) or UAN (4 to 8 pt/A). Use a silicon-based surfactant with the Galaxy tank mix (0.25% v/v).

Comments

- Application in tank mixes may reduce grass control.
- Treated fields may be used for hay but not for grazing or forage.
- Do not apply to soybeans within 75 days of harvest.
- Do not apply if rainfall is expected within one hour following application.

Trade Name	Common Name	Formulation	Rate/Range
Pursuit	imazethapyr	2 AS	4 oz/A
Pursuit EcoPak		70 DG	1.44 oz/A (1 bag/5A)

Mode of Action

ALS

Application Timing

Apply when weeds are actively growing and before weeds exceed maximum size specified on label. For most species, maximum size is 3 inches.

Recommended Spray Additives

Nonionic surfactant-1 qt/100 gal, or crop oil concentrate-1% (v/v), or methylated seed oil-1% (v/v), and include 1 to 2 qt/A nitrogen-based liquid fertilizer or 2.5 lb/A ammonium sulfate.

Comments

- Pursuit may be applied preplant incorporated or preemergence up to 45 days ahead of planting. Incorporation improves consistency of soil-applied treatments.
- Pursuit has long soil residual characteristics and may occasionally carry over. Refer to label for specific restrictions.
- Do not apply products containing chlorimuron ethyl, flumetsulam, imazaquin, or imazethapyr the same year as Pursuit.
- Do not graze or feed treated soybean forage, hay, or straw to livestock.
- Pursuit may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Pursuit may interact with ALS herbicides applied to corn the following year, increasing potential for injury to corn.
- Do not tank mix Pursuit with clomazone products.
- Do not apply Pursuit within 85 days of harvest.
- Apply prior to soybean bloom.
- Apply combination of Pursuit plus Roundup Ultra to RR soybean varieties.

Trade Name	Common Name	Formulation	Rate/Range
Raptor	imazamox	1 AS	4 to 5 oz/A

Mode of Action

ALS

Application Timing

Apply postemergence when weeds and soybeans are actively growing and before the weeds exceed a height of 5 inches in height but prior to soybeans reaching the bloom stage of development.

Recommended Spray Additives

Crop oil concentrate-1% (v/v); or nonionic surfactant-0.25% (v/v); or methylated seed oil-1%(v/v). Nitrogen fertilizer solution-Always include a nitrogen-based fertilizer at 1 to 2 qt/A with either a COC or NIS. AMS at 2.5 lb/A may be substituted for 28% N or 10-34-0.

Rates for Combinations with Raptor

Raptor	Combination Product	Rate/Range
4 to 5 oz/A	Blazer	8 to 20 oz/A
4 to 5 oz/A	FirstRate	0.15 to 0.3 oz/A
4 to 5 oz/A	Flexstar HL	10 to 20 oz/A
4 to 5 oz/A	Roundup Ultra	16 to 32 oz/A

Comments–Raptor

- Use a soil-applied herbicide for grass control if a heavy infestation of annual grasses is present.
- Allow at least 85 days between application and soybean harvest.
- Raptor provides shorter residual control than Scepter or Pursuit.
- Do not apply after soybeans have initiated flowering.
- Raptor may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Do not make more than one application per year.
- Do not graze or feed treated soybean forage, hay, or straw to livestock.
- Tank mixing Raptor with selective postemergence grass and broadleaf herbicides may reduce grass control. A sequential program is recommended.
- Do not use liquid fertilizer as a carrier.
- Do not apply through any type of irrigation system.
- For maximum weed control, cultivate 7 to 10 days following application.

Trade Name	Common Name	Formulation	Rate/Range
Resource	flumiclorac	0.86 EC	4 to 12 oz/A

Mode of Action

PPO

Application Timing

Apply postemergence to actively growing weeds within size recommendations.

Recommended Spray Additives

COC-1 qt/A; 28% N or AMS may be used in addition to COC for control of tall velvetleaf.

Comments

- Use 12 oz rate for late control of large velvetleaf.
- Do not apply within 60 days of harvest or after pod set.
- Do not graze treated fields or harvest for forage or hay.

Trade Name	Common Name	Rate/Range
Rezult	Rezult B (bentazon 4 lb/gal) Rezult G (sethoxydim 1 lb/gal)	3.2 pt/A

Mode of Action

PSII + ACCase

Application Timing

Apply when weeds are small and actively growing. Refer to the label for specific size restrictions.

Recommended Spray Additives

UAN (1 to 2 qt/A); COC (1 pt/A); DASH (1 pt/A); or ammonium sulfate (1 to 2 lb/A).

Comments–Result

- Registered for aerial application.
- Do not apply if rainfall is expected within one hour following application.
- Do not apply to weeds or crops that are under stress.
- Do not apply within 75 days of harvest.
- Do not graze or cut treated soybean fields for hay for at least 30 days after application.

Trade Name	Common Name	Formulation	Rate/Range
Roundup Ultra	glyphosate	3SL	24 to 48 oz/A
Roundup UltraMax		4SL	20 to 40 oz/A
Roundup UltraDry		65DG	9.5 to 28.8 oz/A
Roundup WeatherMAX		5.5L	16 to 32 oz/A
Roundup WeatherMax		4.5 SL	22 to 44 oz/A
Many others		3SL	24 to 48 oz/A
Touchdown IQ		3SL	1 to 4 pt
Touchdown HiTech			
Touchdown Total			

Mode of Action

EPSPS

Application Timing–Roundup

Postemergence - Glyphosate may be applied from cracking through full flowering stage.

Recommended Spray Additives

AMS may be included if weeds are under stress or hard water is used as carrier. Certain glyphosate products recommend the use of a nonionic surfactant.

Comments

- Use only on Roundup Ready® soybean varieties.
- Refer to label of product being used to determine specific recommendations for rates, spray additives, and restrictions.
- Multiple applications can be made during the growing season. Do not exceed 2 qt/A Roundup Ultra with postemergence applications. A total of 8 qt/A may be applied during the growing season, including preplant, preemergence, postemergence, and preharvest applications.
- Do not apply aerially.
- Take appropriate precautions to minimize off-target movement of spray droplets.
- The timing and rates of glyphosate used for annual weeds will not be optimal for most perennial weeds.
- Equivalent rates of different concentration formulations of glyphosate

Roundup Original (3 lb ae/gal)	Roundup Ultramax (4 lb ae/gal)	Roundup WeatherMax (4.5 lb ae/gal)
24 oz	20 oz	16 oz
32 oz	26 oz	22 oz
40 oz	32 oz	27 oz

Trade Name	Common Name	Formulation	Rate/Range
Scepter	imazaquin	70DG	1.4 to 2.8 oz/A

Mode of Action

ALS

Application Timing

Postemergence—Apply postemergence to actively growing weeds within size recommendations.

Preplant incorporated—Apply preplant incorporated before planting and incorporate into the top 1 to 2 inches of soil. May be applied up to 45 days prior to planting. Incorporate within 7 days of application.

Preemergence—Apply preemergence up to 45 days before, during, or after planting but before the crop emerges.

Postemergence—Apply postemergence to actively growing weeds within size recommendations.

Recommended Spray Additives

When used alone, apply with COC, nonionic surfactant, or organo-silicone surfactant. See label for specific recommendations.

Comments

- Scepter has long soil residual characteristics and has demonstrated carryover to rotational corn.
- Do not graze or feed treated soybean forage, hay, or straw to livestock.
- Do not apply the Scepter + Prowl combination if soybeans have emerged.
- Iowa State University does not recommend combinations with 2,4-DB or 2,4-D amine.
- Iowa State University recommends the use of Clearfield hybrids following a postemergence application of Scepter.
- Refer to Scepter label for specific rotational recommendations.
- IMI corn may be planted after 9.5 months following application of Scepter + Pursuit applications.
- Scepter may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Do not use products containing chlorimuron ethyl, flumetsulam, imazethapyr, or imazaquin the same year as Scepter application, unless labeled otherwise, due to carryover to rotational corn.
- There is an 18-month rotational period for non-Clearfield hybrids following a 2.8 oz/A Scepter application.

Trade Name	Common Name	Formulation	Rate/Range
Select/Arrow	clethodim	2 EC	4 to 16 oz/A

Mode of Action

ACCase

Application Timing

Apply postemergence to actively growing grasses according to the Select label. Do not cultivate 7 days prior to or within 7 days after application. Apply prior to 60 days before harvest.

Recommended Spray Additives

Crop oil concentrate-1% (v/v) (but not less than 1 pt/A); 28% N (1 to 2 qt) or AMS (2.5 lb/A) may be used in combination with the COC.

Comments

- Do not apply more than 32 oz/A per season.
- Do not apply with flood nozzles.
- Do not graze treated fields or feed forage or hay to livestock.
- Do not apply if rain is expected within 1 hour of application.
- Apply 4 to 6 oz/A Select with glyphosate to control RR volunteer corn.

Trade Name	Common Name	Formulation	Rate/Range
Stellar	0.7 lb/gal flumiclorac + 2.4 lb/gal lactofen	3.1 EC	5 to 7 oz/A

Mode of Action

PPO + PPO

Application Timing

Apply postemergence to actively growing weeds within size restrictions on label.

Recommended Spray Additives

COC or methylated seed oil (1 to 2 pts/A); 28%N (1 to 2 qts/A); or AMS (2.5 lbs/A) may be used in combination with the COC or MSO to enhance weed control.

Comments

- Do not apply aerially.
- Do not apply after pod set or within 60 days of harvest.
- Do not use hay or straw for feed or bedding.
- Use RR soybean varieties for Stellar plus glyphosate applications.
- The addition of 28% N or AMS with Stellar will increase the potential for crop injury.

Trade Name	Common Name	Formulation	Rate/Range
Synchrony STS	31.8% chlorimuron ethyl 10.2% thifensulfuron methyl	42 DF	0.5 oz/A

Mode of Action

ALS + ALS

Application Timing– Synchrony STS

Apply to actively growing weeds within the recommended growth stage. Apply after the first trifoliolate has fully expanded but no later than 60 days before harvest.

Recommended Spray Additives

Crop oil concentrate-1% (v/v) plus UAN (2 to 4 qt/A); 10-34-0 (1 to 2 qt/A) or ammonium sulfate (2 to 4 lb/A); NIS - 0.25% (v/v) with Blazer and 0.5% (v/v) with Cobra tank mixes; include UAN (2 to 4 qt/A) or ammonium sulfate (2 to 4 lb/A).

Comments

- Can be used on soybean varieties designated as STS in the variety name and non-STS varieties.
- Synchrony STS can be applied to non-STS soybean varieties. Use 0.25 oz/A and NIS at 0.25% (v/v) with UAN or AMS.
- Refer to label for soil pH and rotation restrictions.
- Apply in a minimum of 10 gal. water and use flat fan nozzles.
- Do not graze treated fields or harvest for forage or hay.
- Synchrony may interact with other ALS herbicides applied to soybeans, thus increasing the potential for crop injury.
- Chlorimuron may interact with ALS herbicides applied to corn the following year, increasing potential for injury to corn.
- Iowa State University does not recommend 2,4-DB.
- Apply prior to 60 days before soybean maturity.
- Make only one application per growing season.
- Apply after the first trifoliolate has fully expanded.
- Do not apply with chemigation systems.

Comments—Synchrony STS, continued

- Cobra may be applied in combination with Synchrony STS at a rate of 8 to 12.5 oz/A.
- Use the 4 to 6 oz/A Cobra rate for control of ALS-resistant common waterhemp and black nightshade; use 0.5% COC.
- Apply when common waterhemp is actively growing and less than 4 inches tall.
- Chlorimuron has a long soil residual life in soils with a high pH (7.0 or greater); refer to the label for specific restrictions concerning crop rotational intervals.
- Soils of concern for carryover are those in the Clarion-Nicollet-Webster and Hamburg-Ida-Monona associations, and soils in the Missouri River flood plain. Also, fields near limestone gravel roads may have a higher pH than typically anticipated.

Preplant and Preemergence Herbicides for Corn Production

The following herbicides are used to control weeds as they emerge. Certain products require mechanical incorporation prior to planting, while others may be applied before, at, or after planting. Incorporation reduces the need for timely rainfall after application and may improve control of certain weeds. Adjust rate to soil type as specified on label. In no-till, an additional burndown herbicide may be required to control emerged weeds. Many herbicides used primarily for preemergence weed control may also be applied following crop emergence. Recommendations for post applications of these products are included in this section rather than in the postemergence section.

Trade Name	Common Name	Formulation	Rate/Range
AAtrex, atrazine	atrazine	4 L	2 to 4 pt/A
AAtrex, atrazine		90 DF	1.8 to 2.2 lb/A

Mode of Action

PSII

Application Timing—AAtrex, atrazine

Early preplant—Atrazine may be applied as a single application up to 30 days ahead of planting or as a split application up to 45 days ahead of planting.

Preplant incorporated—For best results, apply within 2 weeks prior to planting.

Preemergence—Apply during or shortly after planting before weed emergence.

Postemergence—Apply before broadleaf weeds exceed 4 inches, grasses exceed 1.5 inches, and corn 12 inches in height. Include crop oil concentrate or crop oil.

Comments

- All products containing atrazine are restricted-use pesticides.
- Products containing atrazine include a groundwater advisory statement on the label.
- Atrazine residues may persist and cause damage to susceptible crops the following growing season. See label for rotation restrictions. Carryover is greatest on soils with alkaline pH.
- May be used on ear corn, silage corn, sweet corn, and popcorn.
- Maximum annual rate for preemergence application on highly erodible soils is 2.0 lb a.i./A on fields with 30% or more crop residue and 1.6 lb a.i./A on fields with less than 30% crop residue.
- Maximum annual preemergence rate on soils not highly erodible is 2.0 lb a.i./A.
- Maximum annual postemergence rate where no soil-applied atrazine was used is 2.0 lb a.i./A, and where soil-applied atrazine was used, the total amount of atrazine may not exceed 2.5 lb a.i./A.
- Do not mix or load within 50 feet of any well, sinkhole, intermittent streams and rivers, or natural or impounded lakes and reservoirs.
- No application within 66 feet of the points where field surface water runoff enters perennial or intermittent streams and rivers.

Critical Period of Weed Control in No-Till Soybean (*Glycine max*) and Corn (*Zea mays*)

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Critical Period of Weed Control in No-Till Soybean (*Glycine max*) and Corn (*Zea mays*)¹

CHRIS HALFORD, ALLAN S. HAMILL, JOHN ZHANG, and COLLEEN DOUCET²

Abstract: The critical period of weed control for crops grown under conventional tillage systems has been well studied, and the results generated by these studies have been proven to be very useful in developing ecologically and economically sound weed management practices. However, these management systems may not be directly applicable under no-till situations because the species composition, total amount, and temporal pattern of seedling emergence change substantially with tillage. The objective of this study was to identify the critical period of weed control for soybean and corn in fields that had been under no-till management for 1 yr. Although estimates of the critical period for a crop vary from year to year and site to site, some interesting comparisons can be made between no-till and conventional tillage. The start of the critical period in no-till corn was stable, usually beginning at the six-leaf stage. The end of the critical period was more variable ranging from the 9- to 13-leaf stage. The critical period for corn under no-till conditions tended to start and end earlier than under conventional tillage practices. In soybean, we were unable to identify a critical period at one of the sites. At the other location (sandy loam soil), the critical period was estimated to begin at the first or second node developmental stage, whereas the end was determined to be at the R1 stage (early flowering). The critical period in soybean was longer than that observed under conventional tillage.

Nomenclature: Corn, *Zea mays* L. 'Pioneer 3573'; soybean, *Glycine max* (L.) Merr. 'Northrup King 2492'.

Additional index words: Weed interference, crop yield loss.

Abbreviation: DAE, days after emergence.

INTRODUCTION

A combined effort of minimizing the level of interference to the natural environment (Hildebrand 1990; Reganold et al. 1990) and reducing the labor and fuel input to maintain profitable crop production (Brown et al. 1989; Griffith et al. 1986; Hairston et al. 1984) has promoted the adoption of no-till agricultural systems (Klassen 1991; Swanton and Weise 1991). This economically and ecologically sound management system has been gaining popularity, in many areas of the world, in recent years (Swanton and Weise 1991). A great deal of evidence has shown that conservation tillage systems can be more productive than conventional tillage systems

(Shelby et al. 1988; Wilcox-Lee and Drost 1991) as a result of improved soil quality and water use efficiency of the plants (Griffith et al. 1986; Hairston et al. 1984). However, weed control in no-till systems is a major problem that has been purported to require a heavy reliance on herbicides (Buhler 1988; Coffman and Frank 1991; Koskinen and McWhorter 1986; Nowak 1983).

The few available effective weed control procedures in no-till systems may be partially a result of the inapplicability of the available techniques developed under conventional tillage systems. A switch from conventional to conservation tillage systems will alter the species composition, total amount, and temporal pattern of emergence of weeds, and thus result in a modified weed-crop relationship that is not normally observed in conventional tillage systems (Buhler 1995; Doll et al. 1992). In general, weed seedlings tend to emerge later, but at a greater density in no-till as compared with conventional tillage systems (C. Swanton, personal communication; Zhang and Hamill, unpublished data). These are perhaps a result of low soil temperature associated with no-till (Fortin and Hamill 1994). Another tendency observed

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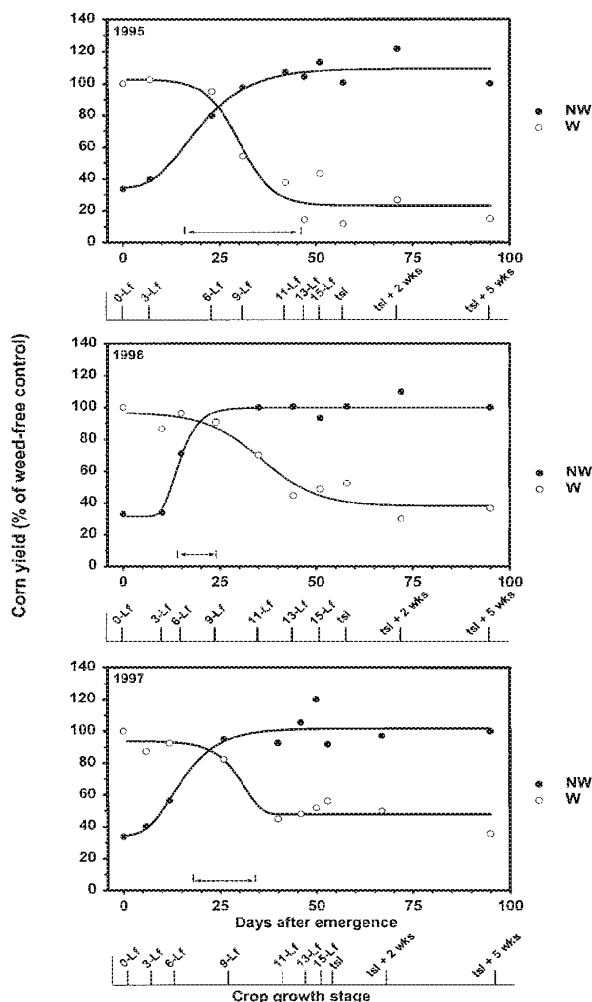


Figure 1. The critical period of weed control in corn and mean yield of corn grain over various weedy (W) and weed-free (NW) treatments at various DAE and crop growth stages in 1995, 1996, and 1997 on fine-textured soil at Wood-see, Ontario. Points represent observed mean values; lines represent the fitted curves.

under no-till conditions is the domination of annual grasses in the weed population (Johnson et al. 1998). For example, green foxtail [*Setaria viridis* (L.) Beauv.] shows a greater density and longer period of emergence under no-till conditions than under conventional tillage conditions (Buhler 1995). The immediate impact of these results is an altered strength and pattern of competition between weeds and crops, because both density and order of emergence of plants are determinants of compe-

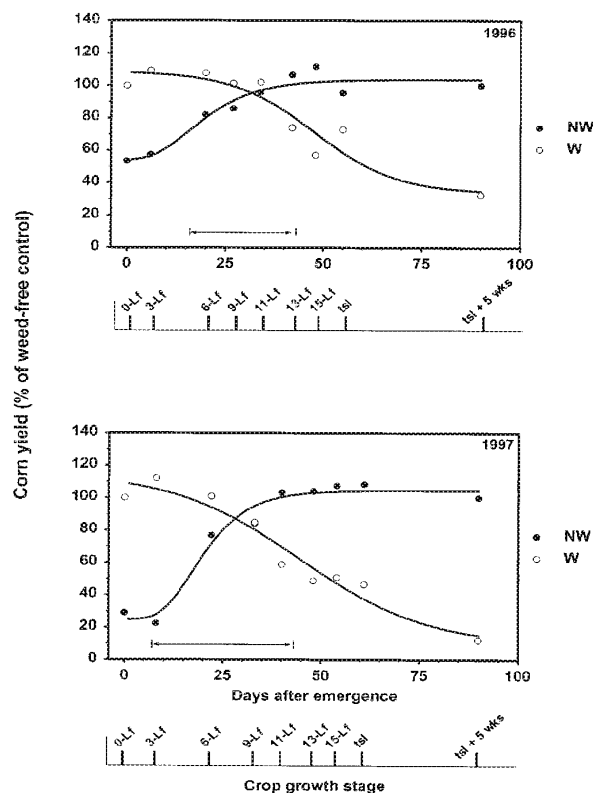


Figure 2. The critical period of weed control in corn and mean yield of corn grain over various weedy (W) and weed-free (NW) treatments at various DAE and crop growth stages in 1996 and 1997 on coarse-textured soil at Harrow, Ontario. Points represent observed mean values; lines represent the fitted curves.

tion success (Harper 1977; Weaver and Cavers 1978). It is thus reasonable to expect that crops grown in conventional and no-till systems may differ in the critical period of weed control. Current methods of weed control, especially herbicide application, developed for conventional tillage systems may not suit the biological necessity (weed population) under no-till situations (Van Acker et al. 1993). Improvements in the efficacy of these methods can be achieved through the identification of the critical period of weed control in no-till systems.

The critical period of weed control refers to an interval in the life cycle of the crop when weed interference will cause yield loss of the crops (Zimdahl 1980). Extensive studies have been conducted to identify the critical period of weed control in various crops under conventional tillage systems (Hall et al. 1992; Van Acker et

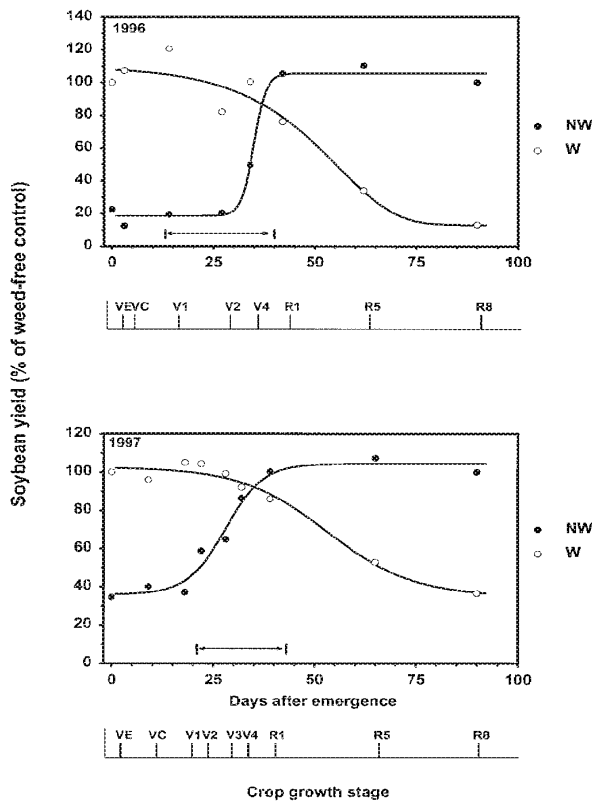


Figure 3. The critical period of weed control in soybean and mean yield of soybean over various weedy (W) and weed-free (NW) treatments at various DAE and crop growth stages in 1996 and 1997 on fine-textured soil at Harrow, Ontario. Points represent observed mean values; lines represent the fitted curves.

al. 1993; Weaver and Tan 1983). Most of them show that there indeed is a period of time when weed interference affects crop yield. For example, 9 to 38 d after emergence (DAE) was reported to be the critical period of weed control for soybean grown in conventional tillage system (Van Acker et al. 1993). If weed management is maintained during this period, the efficiency of the program can be greatly enhanced, or the intensity or frequency of application of that program can be reduced (Hall et al. 1992; Van Acker et al. 1993). Little effort has been made to identify the critical period of weed control in no-till systems, although the total area of no-till crops has increased greatly in recent years (Klassen 1991). Various questions remain unanswered, for example, will the delayed seedling emergence of weeds at a great density under no-till systems alter the critical

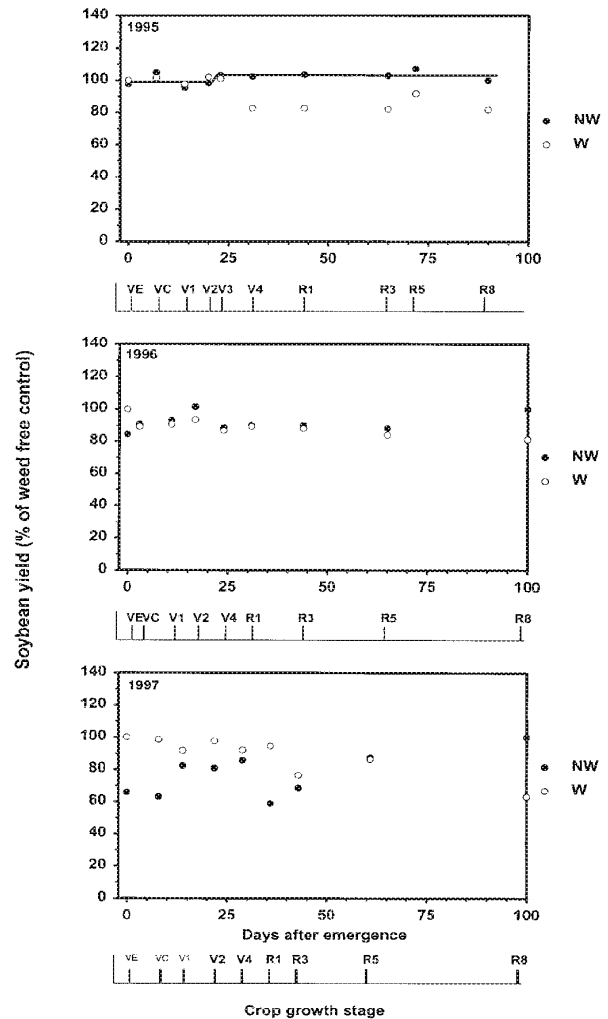


Figure 4. The critical period of weed control in soybean and mean yield of soybean over various weedy (W) and weed-free (NW) treatments at various DAE and crop growth stages in 1995, 1996, and 1997 on fine-textured soil at Woodslee, Ontario. Points represent observed mean values; lines represent the fitted curves.

period of weed control. Another question that remains to be answered is how the current techniques of weed control, developed under conventional tillage systems, have to be modified to account for the variation in weed emergence. The reduction of herbicide application in no-till systems relies largely on the identification of the critical period of weed control in various crops. This can be achieved by examining the effect of the length of the

weed-free and weed-interference periods on yield production of a given crop. The experimental procedure has been well established (Hall et al. 1992; Van Acker et al. 1993).

The purpose of this study is to identify the critical period of weed control in no-till soybean and corn, the two major field crops (in economic terms) in Ontario, Canada. It is expected that the results may help to develop weed control techniques to reduce herbicide application in no-till systems.

MATERIALS AND METHODS

Study Sites and Information. Field experiments were conducted on corn (Pioneer 3573) and soybean (Northrup King 2492) crops in 1995, 1996, and 1997 at the Agriculture and Agri-Food Canada Eugene F. Whelan Experimental Farm at Woodslee, Ontario (42°11'N, 82°42'W), and in 1996 and 1997 at the Agriculture Canada Research Centre in Harrow (42°02'N, 82°55'W), Ontario, Canada. The soil type in Harrow is a Fox sandy loam (Hapludalf subgroup; sand 83%, silt 5%, clay 13%, organic matter 2.6%; pH 6.4). The total area of the field at the Harrow site was 2.17 ha (38 m by 57 m). Corn and soybean had been planted in 3-m-wide alternating strips within the field since 1990. The corn and soybean strips were rotated each year. The soil type in Woodslee is a Brookston clay loam (humic gleysol; sand 36%, silt 30%, clay 35%, organic matter 4.1%; pH 6.5). At Woodslee, corn and soybean crops were grown in separate areas within a field. No-till corn was grown in a 1.84-ha (25.5 m by 72 m) area, while no-till soybean was grown in a 0.89 ha (25.5 m by 35 m) area. One year of no-till and continuous corn or soybean had been grown in each area prior to the start of this study.

Experimental Procedure. The selected fields in Woodslee and Harrow were divided into four blocks, each serving as a replicate. At Harrow, each block contained two rows of 18 consecutive plots measuring 3 m by 3.5 m, where plots were alternately sown with corn and soybean to follow a previously established pattern of corn and soybean rotation. At Woodslee, each block in both the corn and soybean field was divided into 20 consecutive plots measuring 2.15 m by 8 m in the corn area and 1.125 m by 8 m in the soybean area. Before planting, each field was treated with glyphosate at 0.9 and 1.2 kg ai/ha for the Harrow and Woodslee experiments, respectively. After the initial glyphosate burn-down, each field was overseeded on an average with 1 kg/ha of a mixture of locally collected weed seeds, composed mainly of

Table 1. Corn developmental growth stages and corresponding days after crop emergence in which weeds were allowed (W) or not allowed (NW) to interfere.

Treatment	Growth stage	Days after emergence ^a				
		Harrow		Woodslee		
		1996	1997	1995	1996	1997
W0, NW0	0-leaf(LF) (emergence)	0	0	0	0	0
W3, NW3	3-LF ^b	6	8	7	10	6
W6, NW6	6-LF ^b	20	22	23	15	12
W9, NW9	9-LF ^b	27	33	31	24	26
W11, NW11	11-LF ^b	34	40	42	35	40
W13, NW13	13-LF ^b	42	48	47	44	46
W15, NW15	15-LF ^b	48	54	51	51	50
WT, NWT	Tasseling	55	61	57	58	53
WT2, ^c NWT2 ^c	Tasseling + 2 wk			71	72	67
WT5, NWT5	Tasseling + 5 wk	90	96	95	93	88

^a Days after emergence indicate "weed removal days."

^b Leaves emerged from the whorl.

^c Woodslee site only.

foxtails, *Setaria* spp.; common ragweed, *Ambrosia artemisiifolia* L. #³ AMBEL; velvetleaf, *Abutilon theophrasti* Medik. # ABUTH; pigweed, *Amaranthus* sp.; common lambsquarters, *Chenopodium album* L. # CHEAL. The overseeding took place every year at both locations. The crops were planted at depths between 35 and 40 mm using a Kinze MaxEmerge precision planter.⁴ Corn was planted at a rate of 68,710 seeds/ha and soybean at 80.64 kg/ha. At Harrow, corn and soybean were planted in 0.75-m-wide rows, with four rows per plot. Both crops were planted on May 6, 1996 and May 5, 1997. At Woodslee, corn was planted in 0.75-m-wide rows, with three rows per plot; soybean was planted in 0.375-m-wide rows, with three rows per plot. Corn was planted on June 1, 1995, May 29, 1996, and June 11, 1997, whereas soybean was planted on June 1, 1995, May 31, 1996, and June 19, 1997.

Prior to crop emergence, each of the 20 (Woodslee) or 18 (Harrow) plots within each block was randomly selected to receive one of the treatments listed in Table 1 (corn) and Table 2 (soybean). The treatments created two groups of plots that were kept weedy or weed free for various lengths of time after crop seedling emergence, and then maintained weed free or weedy for the remainder of the growing season. Plots that were kept weedy or weed free for any given number of days after seedling emergence were referred to as weedy or weed-free plots. The weed-free phase of each plot was maintained by removing weeds around soybean and corn plants by hand and then hoeing between rows with minimum disturbance to the soil sur-

³ Letters following this symbol are a WSSA-approved computer code from *Composite List of Weeds*, Revised 1989. Available only on computer disk from WSSA, 810 East 10th Street, Lawrence, KS 66044-8897.

⁴ Kinze Manufacturing Inc., I-80 North, Williamsburg, IA 52361.

Table 2. Soybean developmental growth stages and corresponding days after crop emergence in which weeds were allowed (W) or not allowed (NW) to interfere.

Treatment	Growth stage ^a	Days after emergence ^b				
		Harrow		Woodslee		
		1996	1997	1995	1996	1997
WVE, NWVE	Emergence	0	0	0	0	0
WVC, NWVC	Cotyledon	3	9	7	3	8
WV1, NWV1	First node	14	18	14	11	14
WV2, NWV2	Second node	27	22	20	17	22
WV3, ^c NWV3 ^c	Third node		28	23		
WV4, NWV4	Fourth node	34	32	31	24	29
WR1, NWR1	Beginning flower	42	39	44	31	36
WR3, ^d NWR3 ^d	Beginning pod			65	44	43
WR5, NWR5	Beginning seed	62	65	72	65	61
WR8, NWR8	Full maturity	100	100	90	100	100

^a Soybean growth stages are listed according to plant vegetative (V) and reproductive (R) development.

^b Days after emergence indicate "weed removal days."

^c Harrow site in 1997 only.

^d Woodslee site only.

face. Crop plants in the season-long, weed-free plots were used to determine the growth stages of plants when weeds had to be removed from a given plot (Tables 1 and 2) (Fehr and Caviness 1997; Hall et al. 1992). Within each year, treatments were rerandomized among plots within each block.

Data Collected. Weeds found within two 0.25 m by 0.25 m quadrats were identified and counted in the fall of 1996 and 1997 in each of the season-long weedy plots at both the locations and in both the crops. Mean values of total weeds and each individual weed species calculated across the four replicates were used to estimate the potential weed density in the field. At the end of each growing season, the middle two rows of each corn and soybean plot were combine harvested from the Harrow site. At Woodslee, the middle row of each corn and soybean plot was combine harvested. Yield per plot was then converted to tonnes per hectare. The mean yield (t/ha) per treatment for each crop was adjusted for a standard moisture level of 15.5% for corn and 14.0% for soybean.

Statistical Analysis. Soybean and corn were analyzed separately and identically. Mean yield across the four blocks was calculated for each treatment and then converted to percentage values (relative yield) of the season-long weed-free control in each treatment group. The calculated relative yields for the weedy or weed-free treatments were regressed against the number of days for which the plots were kept weedy or weed free using nonlinear regression following a four-parameter sigmoid model. For the weedy plots, leaving weeds in the field

up to a certain point of time early in the season would not affect soybean yield. After that, drastic yield reduction occurred if the plots were not kept weed free. Similarly, for the weed-free plots, keeping the field weed free after a certain point of time later in the season would not improve soybean yield. Prior to that point, drastic yield reduction occurred if the plots were not kept weed free. By choosing a 2.5% yield reduction as a threshold, the dates before or after which weed control was not essential to yield were determined. The time period between these two dates was considered the critical period of weed control. Several functions generate sigmoidal curves. The two that are traditionally used to determine critical periods of weed control are the logistic and the Gompertz equations (Hall et al. 1992, Van Acker et al. 1993). These equations were screened for their fitness of the data (r^2):

$$Y = d + a \times \exp(-\exp(-(X - c)/b)) \quad [1]$$

$$Y = a/(1 + \exp(b \times (X - c))) + d \quad [2]$$

where for both equations: Y is the yield (% of season-long weed-free control), X the days after crop emergence, a the range between the maximum and minimum mean yield (%), c the point of greatest inflection (days), b the slope of the curve at the point of greatest inflection, d the minimum mean yield (%), and exp the mathematical constant e. The selection of the appropriate function was based on the percentage of variance explained (R^2), the satisfaction of the normal distribution of errors, and homogeneity of residuals.

RESULTS AND DISCUSSION

Weed Densities. In the corn plots (Table 3) total weed densities were similar between locations and years, with an overall average of 102.8 plants/m². In 1996 and 1997, foxtails and barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] were the dominant species at Woodslee, while foxtails and common lambsquarters dominated at Harrow. Although all fields were overseeded each year with weed seeds at the same rate, weed densities in soybean plots differed between years and locations (Table 4). Plots at Harrow had substantially greater weed density than those at Woodslee. The weed community at Woodslee had a wide weed spectrum without an apparently dominating species, whereas that at Harrow was dominated by common lambsquarters, barnyardgrass, and yellow foxtail [*Setaria glauca* (L.) Beauv.]. At Harrow, weed density was lower in 1997 than in 1996 mainly due to a drop in the population size of pigweed, barn-

Table 3. Species composition and density of weeds in corn using the season long weedy plots. Mean density (number of plants/m²) is followed by the standard error.

Species (scientific name)	Woodslee		Harrow	
	1996	1997	1996	1997
Common ragweed (<i>Ambrosia artemisiifolia</i> L.)	3.0 ± 1.5	4.5 ± 1.3	9.5 ± 6.0	7.5 ± 3.1
Ladysthumb (<i>Polygonum persicaria</i> L.)	1.0 ± 0.5	—	0.5 ± 0.4	6.0 ± 2.1
Common lambsquarters (<i>Chenopodium album</i> L.)	8.0 ± 4.7	3.5 ± 3.0	16.5 ± 6.6	41.0 ± 10.3
Pigweed species (<i>Amaranthus</i> sp.)	0.5 ± 0.4	7.5 ± 3.3	13.0 ± 4.0	6.0 ± 3.1
Velvetleaf (<i>Abutilon theophrasti</i> Medik.)	4.5 ± 1.8	9.0 ± 2.9	0.5 ± 0.4	—
Barnyardgrass [<i>Echinochloa crus-galli</i> (L.) Beauv.]	31.0 ± 8.9	54.5 ± 32.5	3.5 ± 3.0	9.5 ± 7.7
Crabgrass species (<i>Digitaria</i> sp.)	—	—	1.5 ± 0.8	0.5 ± 0.4
Fall panicum (<i>Panicum dichotomiflorum</i> Michx.)	3.0 ± 2.1	4.5 ± 1.3	0.5 ± 0.4	—
Foxtail species (<i>Setaria</i> sp.)	67.5 ± 18.4	33.5 ± 12.0	29.5 ± 3.3	26.5 ± 20.1
Unknown	—	—	1.5 ± 0.8	2.0 ± 1.7
Total	118.5	117	76.5	99

yardgrass, and yellow foxtail. These three weeds require a relatively high germination temperature in spring, which might not have been satisfied in 1997 because of low mean daily temperatures (data not shown). In other studies, as tillage was reduced the most commonly reported change in annual weed populations was the increase in summer annual grass species such as giant foxtail (*Setaria faberi* Herrm.), green foxtail, yellow foxtail, fall panicum (*Panicum dichotomiflorum* Michx.), and field sandbur [*Cenchrus longispinus* (Hack.) Fern.] (Buhler 1995).

Crop Yield and Critical Periods in Corn. Results for the corn yield data showed that a four-parameter Gompertz function best fit the weed-free period data for all the years and both the sites. The four-parameter logistic function provided the best fit for the weedy period data (critical time for weed removal), with the exception of Woodslee in 1997. Equation parameters are given in Table 5. At Woodslee, the start of the critical period was

relatively stable among years, with 16, 14, and 18 DAE in 1995, 1996, and 1997, respectively (Figure 1), all of which correspond to approximately the six-leaf stage. The end of the critical period was more variable ranging from 24 to 46 DAE, or the 9- to 13-leaf crop stage. The critical period observed at Harrow in 1996 was similar to those seen at Woodslee, with 16 to 43 DAE or the 6- to 13-leaf stage (Figure 2). The critical period at Harrow in 1997 began relatively early at 7 DAE, or the three-leaf stage. The onset and the duration of the critical period at Harrow were greatly influenced by climatic factors. The spring of 1997 was cooler and wetter than that of 1996 (data not shown). The weather was cold enough in 1997 to delay corn emergence by 1 wk, but warm enough to allow early season weed species like common lambsquarters to emerge. Wet weather during the same period allowed for a higher density of larger weeds, such as common lambsquarters, to be present when corn finally emerged.

Table 4. Species composition and density of weeds in soybean using the season long weedy plots. Mean density (number of plants/m²) is followed by the standard error.

Species (scientific name)	Woodslee		Harrow	
	1996	1997	1996	1997
Annual sowthistle (<i>Sonchus oleraceus</i> L.)	9 ± 6.69	—	—	—
Common ragweed (<i>Ambrosia artemisiifolia</i> L.)	1.5 ± 0.43	2 ± 1	1 ± 0.5	5 ± 3.2
Dandelion (<i>Taraxacum officinale</i> Weber)	7.5 ± 1.3	10.5 ± 3.96	—	—
Eastern black nightshade (<i>Solanum ptycanthum</i> Dun.)	—	0.5 ± 0.43	—	—
Ladysthumb (<i>Polygonum persicaria</i> L.)	0.5 ± 0.43	—	3.5 ± 0.43	3.5 ± 2.49
Common lambsquarters (<i>Chenopodium album</i> L.)	4 ± 1.87	0.5 ± 0.43	23 ± 9.6	48 ± 31.87
Pigweed species (<i>Amaranthus</i> sp.)	4.5 ± 3.34	7 ± 0.5	30.5 ± 6.83	8.5 ± 3.27
Shepherd's-purse [<i>Capsella bursa-pastoris</i> (L.) Medi]	1.5 ± 1.3	—	—	—
Velvetleaf (<i>Abutilon theophrasti</i> Medik.)	9 ± 1.66	23 ± 7.16	2 ± 1	2 ± 1.22
White clover (<i>Trifolium repens</i> L.)	0.5 ± 0.43	—	—	—
Barnyardgrass [<i>Echinochloa crus-galli</i> (L.) Beauv.]	11.5 ± 6.53	8.5 ± 2.17	58 ± 26.9	17.5 ± 14.58
Crabgrass species (<i>Digitaria</i> sp.)	1 ± 0.87	0.5 ± 0.43	8.5 ± 3.49	11.5 ± 5.76
Fall panicum (<i>Panicum dichotomiflorum</i> Michx.)	0.5 ± 0.43	—	2.5 ± 2.17	—
Foxtail species (<i>Setaria</i> sp.)	3 ± 0.87	7 ± 2.96	131.5 ± 38.58	17 ± 3.64
Unknown	2.5 ± 0.8	—	2.5 ± 1.3	1 ± 0.5
Total	54 ± 9.41	59.5 ± 6.76	263 ± 72.32	114 ± 43.15

Table 5. Parameter estimates for equations used to predict critical periods.

Crop	Location	Year	Treatment	Equation ^a	a	b	c	d	r ²
Corn	Woodslee	1995	No weeds	Gompertz ^b	78.2	-0.16	19.5	30.3	0.996
			Weedy	Logistic ^c	79.6	0.23	30.1	23.1	0.977
		1996	No weeds	Gompertz	68.3	3.33	13.3	31.6	0.997
			Weedy	Logistic	58.8	0.14	35.8	38.0	0.992
		1997	No weeds	Gompertz	67.4	6.59	12.4	34.3	0.993
			Weedy	Gompertz	45.6	1.64	26.7	47.7	0.993
	Harrow	1996	No weeds	Gompertz	49.8	9.24	15.9	53.8	0.997
			Weedy	Logistic	75.2	0.10	47.6	33.6	0.991
		1997	No weeds	Gompertz	79.6	7.71	17.2	24.8	0.996
			Weedy	Logistic	106.6	0.06	44.5	9.0	0.991
	Soybean	1995	No weeds	Logistic	4.0	-5.31	22.5	99.2	0.999
			Weedy	No fit	---	---	---	---	---
		1996	No weeds	No fit	---	---	---	---	---
			Weedy	No fit	---	---	---	---	---
		1997	No weeds	No fit	---	---	---	---	---
			Weedy	No fit	---	---	---	---	---
		1996	No weeds	Logistic	86.9	-0.66	34.9	18.6	0.997
			Weedy	Gompertz	96.8	-14.37	55.7	13.1	0.989
		1997	No weeds	Logistic	68.4	-0.22	27.9	36.0	0.997
			Weedy	Logistic	67.6	0.09	53.1	35.1	0.999

^a For both equations: Y is the yield (% of season-long weed-free control), X the days after crop emergence, a the range between the maximum and minimum mean yield (%), c the point of greatest inflection (days), and b the slope of the curve at the point of greatest inflection.

^b $Y = d + a \times \exp(-\exp(-(X-c)/b))$.

^c $Y = a/(1 + \exp(b \times (X-c))) + d$.

Crop Yield and Critical Periods in Soybean. Results for the Harrow soybean yield data showed that a four-parameter logistic function best fit that data in all cases, with the exception of the weedy period data (critical time for weed removal) in 1996 where the Gompertz function provided the best fit. A critical period of 13 to 40 (V1 and R1 stage) and 21 to 44 (V2 and R1 stage) DAE was identified in 1996 and 1997, respectively (Figure 3). It was observed that weed seedlings started to emerge later in 1997 than in 1996 because of lower spring temperatures. Consequently, the beginning of the critical period of weed control was several days later in 1997 as compared with that of 1996. However, the ending of the critical period of weed control did not differ in the two years.

Soybean yields for plots at Woodslee varied randomly in 1996 and 1997 giving no indication of a critical period of weed control (Figure 4). The 1995 data suggested that the plots should be weed free for 21 DAE without losing yield substantially (2.5%). It was not possible to identify the date at which weeds should be removed. Failure to identify the critical period of weed control for the Woodslee experiment was probably due to two reasons. First, soybean plants were planted in 37.5-cm-row width, rather than 75 cm, which may have successfully reduced weed growth in most plots. It was observed during the weed counts conducted in 1996 and 1997 that the existing weeds were usually small and only a few made it through the soybean canopy. Second, planting occurred much later at Woodslee than in Harrow. All fields were

sprayed with glyphosate within 1 wk before planting. Thus, late emerging weeds, which would have otherwise escaped the herbicide, were killed by the burn-down. Glyphosate burn-down of weeds before planting is a standard production practice for no-till or conservation tillage. In addition to narrow row spacing and a later planting, the absence of a critical period for soybean may have been caused by the inability of subsequent flushes of weeds to emerge through the soil surface after late spring and early summer crusting. The soil at Woodslee is a Brookston clay loam. Under conventional tillage at the same site, Van Acker et al. (1993) were unable to identify a true critical period of weed control with soybean over the two years. The lack of a critical period with either no-till or conventional tillage under the same soil type and at the same location suggests a site-specific (soil type) influence on crop-weed competition.

Comparison to Conventional Tillage. This study, along with others (Hall et al. 1992; Van Acker et al. 1993) revealed that estimates of the critical period for a crop vary from year to year and site to site. Despite this variation, interesting comparisons can be made between no-till and conventional tillage. Overall, the onset of the critical period relative to the corn phenological stages was more stable under no-till, three- to six-leaf, than under conventional tillage practices, where the start of the critical period was found to vary from the 3- to 14-leaf stage of corn development (Hall et al. 1992). In

contrast, the end of the critical period occurs earlier, 9- to 13-leaf stage, under no-till practices than under conventional tillage practices, where the critical period ended on an average at the 14-leaf stage. Therefore, although there is much overlap in the critical periods between the two management practices in corn, in general, we do observe a shift to an earlier onset and earlier end to the critical period under no-till. The critical period in soybean was either nonexistent or longer (V1 and R1) than that observed under conventional tillage. The critical period for soybean at Woodstock and Elora, Ontario, under conventional tillage, was between the second node (9 to 21 DAE) and the fourth node stages (21 to 25 DAE) (Van Acker et al. 1993).

Although weed emergence is delayed under no-till conditions, under these cooler conditions weed species that are able to germinate at cooler temperatures would have a competitive advantage over corn, such as a greater access to light and nutrients. Because the studied fields were only under no-till management for 1 yr prior to the initiation of the study, the species composition was similar to what would have been observed under conventional tillage. Most weed species were annuals (Tables 3 and 4). Given a few more years, a no-till field would show a greater representation of winter annuals, biennials, and perennials. If plants from these groups survived the glyphosate burn-down, they would interfere with the crop earlier due to their larger size, which would cause an even earlier shift than under conventional tillage.

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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.

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NOVEL HERBICIDE RESISTANCE GENESCross-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-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 (Agrilience, 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* (AryloxyAlkanooate

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 $\pm$ 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 $\pm$ 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 μ M 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 T₁ *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T₁ family.

[0041] **Figure 21** shows western blot analysis of individual native *AAD-2* (v1)-transformed T₁ *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.

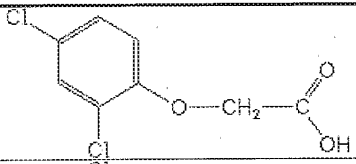
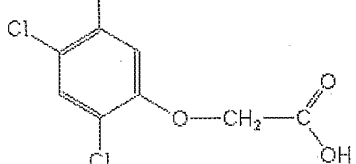
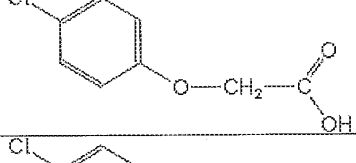
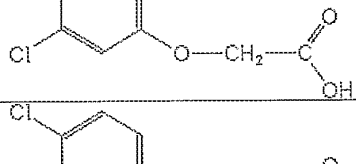
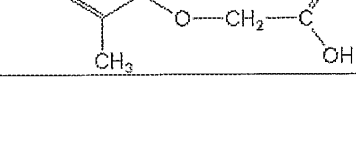
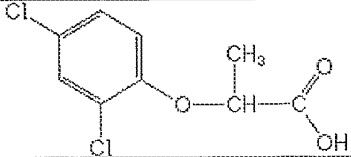
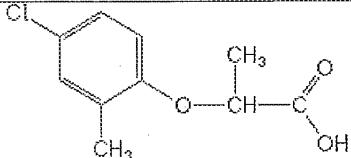
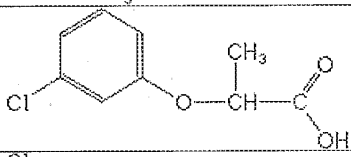
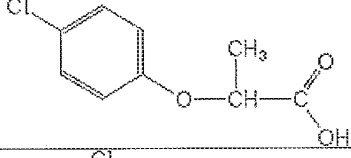
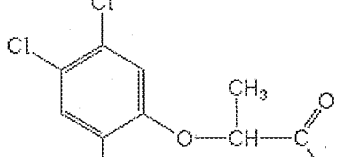
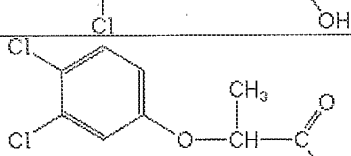
Table 1. Commercially available phenoxy auxins.				
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	
MCPA	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.

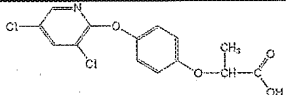
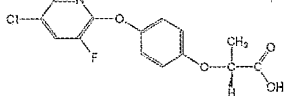
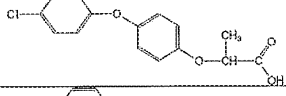
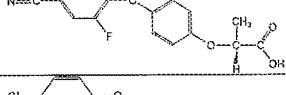
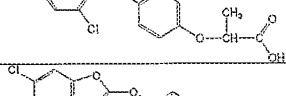
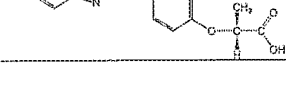
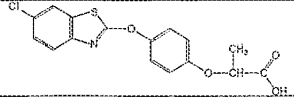
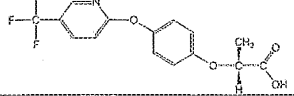
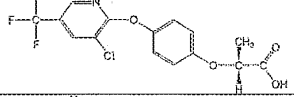
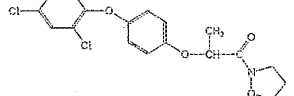
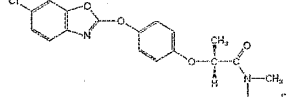
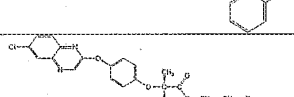
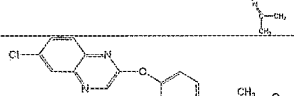
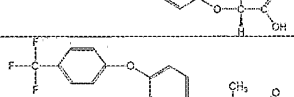
Table 2. AOPP graminicidal compounds listed by accepted common names.				
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	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	
Haloxifop	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, pyrimidinylthiobenzoates, and other chemistries = *AHAS*, *Csr1*, *SurA*, *et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxyphenyl-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-I*), 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 pyriithiobac), 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)X₂₃₋₂₆(T/S)X₁₁₄₋₁₈₃HX₁₀₋₁₃R" 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 aryloxyphenoxypionate 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(NH₄)₂(SO₄)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**).

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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 μ M Fe^{2+} , 200 μ M Na ascorbate, 1 mM α -ketoglutarate using either 1 μ g or 10 μ g (10X) purified AAD-1 (v1) per 0.16 ml assay.

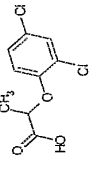
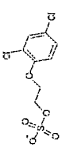
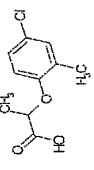
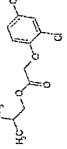
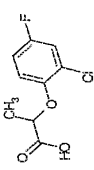
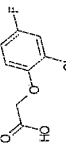
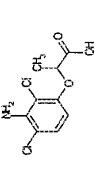
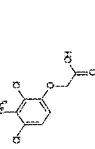
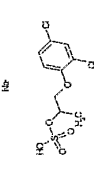
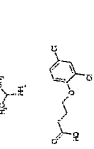
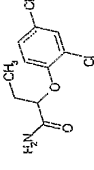
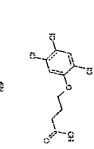
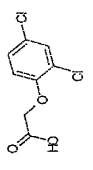
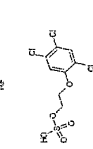
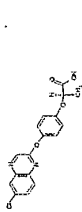
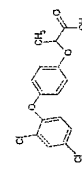
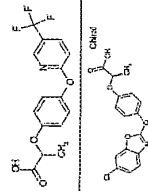
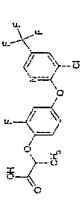
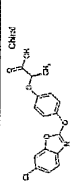
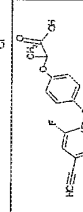
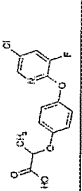
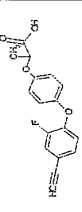
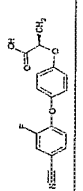
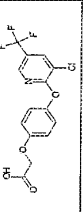
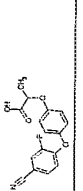
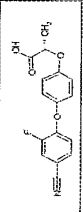
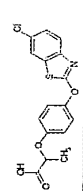
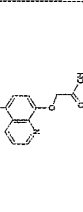
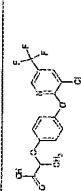
Table 3: Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs									
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)	STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594		398166	sesone	0.02	0.177
	188874	(R,S)-mecoprop	0.341	2.065		190252		0.008	0.211
	83293	(R,S)-2-chloro, 4-fluorophenoxypropionate	0.304	2.358		124988		0.007	0.058
	11113675	(R,S)-3-aminodichlorprop	0.228	2.676		11263526		0.004	0.069
	188476		0.077	0.687		178577		0.003	0.021
	192132		0.064	0.204		178587		0.003	0.02
	195517	2,4-D	0.034	0.383		188527		0.003	0.036

Table 4: Effect of purified AAD-1 (v1) on various AOPP graminicidines 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.

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)	STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalofop	0.43	2.1		460511	(R,S)-diclofop	0.155	1.663
	67131	(R,S)-fluazifop	0.427	2.17		25646	(R,S)-chlorazifop	0.144	1.69
	11044492	(R)-fenoxaprop	0.408	0.597		70222	(R,S)-cyhalofop	0.128	1.584
	34697	(R,S)-clodinafop	0.295	1.98		199608	cyhalofop	0.114	1.26
	14603	(R)-cyhalofop	0.222	1.989		43865	haloxyfop-oxyacetate	0.004	0.053
	14623	(R,S)-cyhalofop	0.215	1.815		7466	(S)-cyhalofop	0.003	0.017
	62942	(R,S)-fenthiaprop	0.199	1.055		204558	Cloquintocet	0	0.001
	66905	haloxyfop	0.172	1.63					

[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 ³²P-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 (T_m) 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):

$$T_m = 81.5^\circ \text{C} + 16.6 \log[\text{Na}^+] + 0.41(\% \text{G+C}) - 0.61(\% \text{formamide}) - 600/\text{length of duplex in base pairs.}$$

[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 T_m-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 (T_m) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T_m for oligonucleotide probes can be determined by the following formula:

$$T_m (^\circ\text{C}) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(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 *Bal31* 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, sulfonyleureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

[00165] In addition to a selectable marker, it may be desirable 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 $\leq 50\%$ 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)* (AryloxyAlkanolate 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% H₂O + 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 μ L 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 KNO₃, 2.5 mM KH₂PO₄, 50 mM FeSO₄, 10 mM NaEDTA (pH 8.0), 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 70 μ M H₃BO₃, 14 μ M MnCl₂, 0.5 μ M CuSO₄, 1 μ M ZnSO₄, 0.2 μ M NaMoO₄·2H₂O, 10 μ M NaCl, 10 nM CoCl₂·H₂O, 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 GR₅₀ 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 μ E/m²s⁻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 GR₅₀ 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)			GR ₅₀ ratio **	Structure
	Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1		
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)			GR ₅₀ ratio **	Structure
	Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1		
Haloxypyr-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 from 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 GR₅₀ 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 GR₅₀ 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 μ M in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μ M NH₄(FeSO₄)₂, 200 μ M 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).

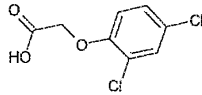
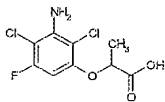
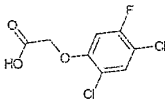
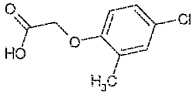
[00208] The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 µg per 160 µl assay and each substrate was tested at 1 mM with an assay time of 15 minutes. **Table 3** shows the A₅₁₀ detected after the 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 μ g) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypyronates relative to auxin phenoxycetates. 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 phenoxycetates.

[00210] Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μ g 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-phenoxycetate 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-dichloroprop	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)-dichloroprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μ M Na ascorbate; 200 μ M 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 μ M), but the k_{cat} values varied significantly. (R)-dichloroprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichloroprop). 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) $\pm$ SE	V_{max} (μmol min⁻¹ mg⁻¹ AAD1) $\pm$ SE	k_{cat} (min⁻¹)	k_{cat}/K_m (min⁻¹ mM⁻¹)
R-dichlorprop	75 $\pm$ 10	0.79 $\pm$ 0.03	26.1	348
R-quizalofop	125 $\pm$ 20	0.57 $\pm$ 0.03	18.9	151
R-haloxypop	120 $\pm$ 54	0.34 $\pm$ 0.04	11.2	94
2,4-D	96 $\pm$ 8	0.57 $\pm$ 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 μ g 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 (Accelrys, 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
Tyrosine	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
	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43		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
22	CGA	0	0.0	0	0	LYS (K)	AAA	1	14.3	2	29
	CGC	16	69.6	5	22		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		TTC	10	76.9	9	69
ASN (N)	AAC	8	100.0	4	50	PHE (F)	TTT	3	23.1	4	31
	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
	GAT	4	21.1	9	47		CCG	7	41.2	0	0
CYS (C)	TGC	3	100.0	2	67		CCT	0	0.0	8	47
	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
	TAG	1	100.0	0	0		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
	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
	GAG	8	50.0	11	69		ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29		ACG	5	21.7	0	0
	GGC	15	71.4	7	33		ACT	1	4.3	8	35
	GGG	3	14.3	2	10	TRP (W)	TGG	5	100	5	100
	GGT	3	14.3	6	29		TAC	7	70.0	6	60
HIS (H)	CAC	6	60.0	5	50	TYR (Y)	TAT	3	30.0	4	40
	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
	ATC	12	100.0	5	42		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:

$$\text{Weighted \% of C1} = 1/(\%C1 + \%C2 + \%C3 + \text{etc.}) \times \%C1 \times 100$$

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
ASN (N)	AAC	50.0	50.0	PHE (F)	TTC	49.2	49.2
	AAT	50.0	50.0		TTT	50.8	50.8
ASP (D)	GAC	38.1	38.1	PRO (P)	CCA	39.8	36.5
	GAT	61.9	61.9		CCC	20.9	19.2
CYS (C)	TGC	50.0	50.0		CCG	DNU	8.3
	TGT	50.0	50.0		CCT	39.3	36.0
END	TAA	DNU	40.7	SER (S)	AGC	16.0	15.1
	TAG	DNU	22.7		AGT	18.2	17.1
	TGA	100.0	36.6		TCA	21.9	20.6
GLN (Q)	CAA	55.5	55.5		TCC	18.0	16.9
	CAG	44.5	44.5		TCG	DNU	6.1
GLU (E)	GAA	50.5	50.5		TCT	25.8	24.2
	GAG	49.5	49.5	THR (T)	ACA	32.4	29.7
GLY (G)	GGA	31.9	31.9		ACC	30.2	27.7
	GGC	19.3	19.3		ACG	DNU	8.3
	GGG	18.4	18.4		ACT	37.4	34.3
	GGT	30.4	30.4	TRP (W)	TGG	100.0	100
HIS (H)	CAC	44.8	44.8	TYR (Y)	TAC	48.2	48.2
	CAT	55.2	55.2		TAT	51.8	51.8
ILE (I)	ATA	23.4	23.4	VAL (V)	GTA	11.5	11.5
	ATC	29.9	29.9		GTC	17.8	17.8
	ATT	46.7	46.7		GTG	32.0	32.0
					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 PromoterRB7 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 RegionZmUbi1 = *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 $\mu\text{mol}/\text{m}^2\text{sec}$ 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 DH5 α 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, 0.044 μ M benzylamino purine (10 μ l/liter of 1 mg/ml stock in DMSO) and 300 μ l/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 T₁ seed (transformed with native [*AAD-1* (ν 2)] or plant optimized [*AAD-1* (ν 3)] gene) was allowed to dry for 7 days at room temperature. T₁ 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 T₁ 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, T₁ plants (cotyledon and 2-4-leaf 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 $\pm$ 5° C, 50 $\pm$ 30% RH, 14 h light:10 dark, minimum 500 μ E/m²s⁻¹ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1* (ν 3) (plant optimized gene) or *AAD-1* (ν 2) (native microbial gene) to provide phenoxy auxin herbicide resistance.

[00256] Random individual T₁ 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. GR₉₉ 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 T₁ 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 T₁ 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, T₁ 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). T₁ transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T₁ 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 T₁ individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

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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 T₁ 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	<i>AAD-1 (v2)</i>	n	84,000	278	0.33%	0.33%	nd ⁴
Glufosinate ¹	<i>AAD-1 (v3)</i>	p	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D ²	<i>AAD-1 (v3)</i>	p	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate ¹	<i>AAD-2 (v1)</i>	n	1,301,500	228	0.018%	0.007 to 0.021%	100%
Glufosinate ¹	<i>AAD-2 (v2)</i>	p	200,000	224	0.11%	0.11%	nd ⁴

¹Glufosinate selection scheme: 280g ai/ha glufosinate applied 5-6 + 10 DAP²2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP³PAT protein expression determined by PAT ELISA strips⁴nd, not determined⁵codon bias, n-native microbial gene, p=plant optimized

[00262] T₁ 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* T₁ transformants. Both genes imparted resistance to individual T₁ *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/CryIF* 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 T₁ 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 T₁ *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 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/CryIF*-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 T₁ *Arabidopsis* response to a range of 2,4-D rates applied postemergence.

% Injury				% Injury	
				Ave	Std Dev
Native AAD-1 (v2) gene					
Averages	<20%	20-40%	>40%		
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
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
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
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
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 T₁ *Arabidopsis* to the phenoxypipronic 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.** T₁ *Arabidopsis* response to a range of dichlorprop 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 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 17. T₁ *Arabidopsis* response to a range of dichlorprop rates applied postemergence.

AAD-1 v3				% 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		
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		
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/CryIF*-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		
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/CryIF				% 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		
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. T₁ seed

transformed with *PAT* and *AAD1* ($\nu 3$) (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* ($\nu 3$) or *PAT* genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

[00270] 6.7 — Heritability.

[00271] A variety of T₁ events were self-pollinated to produce T₂ seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T₂ siblings. Each individual T₂ 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 T₁ families (T₂ 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 T₂ individuals (T₃ seed). Twenty-five T₃ siblings from each of eight randomly-selected T₂ families were progeny tested as previously described. Approximately one-third of the T₂ 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* ($\nu 3$) 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 (T₄ 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 $\mu\text{E m}^{-2} \text{s}^{-1}$). 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* from 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 T₄ *Arabidopsis* (ARBTH).

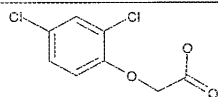
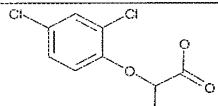
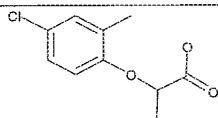
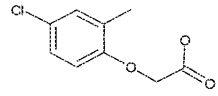
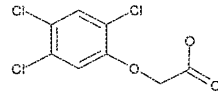
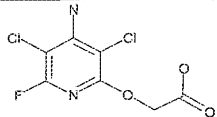
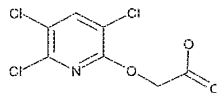
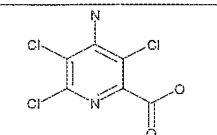
Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Phenoxy auxins							
2,4-D		0.2	10	< 0.01	0.04	50	> 4
dichlorprop		2	5	0.01	1.5	2.5	150
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		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 T₄ *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot 150	PAAD1.315.064 T3 ARBTH shoot 150	wt ARBTH root 150	PAAD1.315.064 ARBTH root 150	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. T₄ 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-dichlorophenol (DCP), on wildtype or transgenic *Arabidopsis*.

Table 20. Comparison of homozygous T ₄ AAD-1 (v3) and wildtype <i>Arabidopsis</i> plant response to various foliar-applied auxinic herbicides.		
Phenoxypropionic auxins		
Herbicide Treatment	Ave % Injury 14DAT	
	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		
Herbicide Treatment	Ave % Injury 14DAT	
	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 <i>Arabidopsis</i> plant response to various foliar-applied auxinic herbicides.		
Pyridyloxyacetic auxins		
Herbicide Treatment	Ave % Injury 14DAT	
	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) T₄ 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 T₄ 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 AGATACCCTTGTTGGTTGC) (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] T₁ *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid (*AAD-1* (v3) + *EPSPS*) coding for a putative glyphosate resistance trait. T₁ 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 T₁ 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* T₅ homozygous lines (pDAB721-transformed), and *PAT* + *CryIF* 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. I₅₀ 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 T₁ plants will be grown to seed, self-pollinated to yield T₂ seed. The pDAB 3230 T₁ plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T₂ 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 DH5 α 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 DH5 α , 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, F₁ 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 AgNO₃, 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 ($13 \mu\text{Em}^{-2}\text{s}^{-1}$) for one week then higher light ($40 \mu\text{Em}^{-2}\text{s}^{-1}$) 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 *Nco*I, a unique cutter, to obtain integration data following probing with *AAD-1* (ν 3), unequivocally demonstrate stable integration of *AAD-1* (ν 3) 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 T₀ plants were regenerated from each of these events and sent for spray testing and seed production.

Table 21.			
Event	PAT Copy #	PCR for <i>AAD-1</i> (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 μ M 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 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 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 T₀ 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 T₀ 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	Haloxypop* 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 T₁ 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 T₁ 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 T₂ events. Plants from individual lineages of each event were randomly self-pollinated in the field. T₂ seed were hand harvested at physiological maturity. Based on single gene copy number (see **Table 24** above) and overall performance in T₁ 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 F₁ 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 T₂ *AAD-1* (*v3*)/*PAT* corn plants were crossed with glyphosate resistant corn plants producing F₁ seed containing *AAD-1* (*v3*), *PAT*, and glyphosate resistance genes as described in the previous example.

[00343] F₁ 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.** T₂BC₁ seeds of hemizygous event number 3404-025.001R/R001 Bulk.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) T₀ corn plants to quizalofop herbicide. A target of approximately eight T₀ 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 T₀ 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 T₀ 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) F₁ 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 ($\sim 1 \times 10^9$ cfu/ml) and 100 μ M 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 , 100 μ M 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-haloxyp 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-haloxyp 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 haloxyp, 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-haloxyp 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 μ M 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 μ M 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 T₀ corn lineages containing *AAD-1* (ν 3) 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* (ν 3) into the genome as described previously. Ten single copy events, as indicated in **Table 26**, were chosen to be regenerated.

Table 26.					
Corn event	<i>In vitro</i> Selection agent (nM)	Southern Copy # (callus) AAD-1	Western Blot (AAD-1) T ₀ Callus	Assessment of T ₀ lineages resistant to 35 g ae/ha quizalofop	
				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 T₀ 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 H₂SO₄. 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 $\mu\text{E}/\text{m}^2\text{s}^{-1}$).

[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 fluorometer (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 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 (Table 27).

Table 27. Tobacco T0 events transformed with pDAB721 (<i>AAD-1(v3)</i> + <i>PAT</i>).				
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 μ g 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] T₀ 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 T₀ 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 T₀ 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 T₁ seed. Two *AAD-1* (*v3*) tobacco lines (event 721(2)-013.010 and 721(3)-008.005) were chosen from the T₀ generation. The T₁ 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 T₀ generation. Improved consistency of response is anticipated in T₁ 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 T₁ 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 T₁ tobacco plants' response to phenoxy auxin herbicides.			
Herbicide	KY160 -- Wildtype	721(2)013.010 (medium tolerance in T₀ generation)	721(3)008.005 (high tolerance in T₀ 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* ($\nu 3$) 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 T₁ or T₂ 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* ($\nu 3$)-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 (Padgett *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 (Cl₂) 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 Cl₂ 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 from 10 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. T₀ plants will be allowed to self fertilize in the greenhouse to give rise to T₁ seed. T₁ plants (and to the extent enough T₀ plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1* ($\nu 3$) and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T₀ plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T₁ seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T₀ and T₁ 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* ($\nu 3$) will be made using T₁ and T₂ 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 OD⁶⁰⁰ was transferred along with 200 μ M acetosyringone (AS) onto a 100x25 sterile Petri plate. EHA105 *Agrobacterium* suspension at a density of 1.2 OD⁶⁵⁰ in a 100 μ L 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 KH₂PO₄ 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 $\mu\text{E}/\text{m}^2$, 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	*	55	1	pos	neg
14	1	pos	**	56	5	pos	*
15	1	pos	*****	57	2	faint pos	neg
16	1	pos	*****	58	8	pos	*****
17	1	pos	*	59	2	pos	*****
18	1	pos	***	60	5	pos	*****
19	1	pos	**	61	1	pos	**
20	1	pos	*****	62	1	pos	**
21	2	pos	*****	63	1	pos	***
22	1	pos	*****	64	1	pos	***
23	1	pos	*****	65	3	pos	*****
24	4	pos	* or neg	66	5	pos	*
25	1	pos	*****	67	6	pos	*
26	1	pos	*****	68	Low DNA	pos	neg
27	low DNA	pos	*****	69	Low DNA	pos	*****
28	low DNA	pos	**	70	low DNA	pos	**
29	low DNA	pos	*****	71	low DNA	faint pos	**
30	17	pos	*	72	low DNA	pos	*****
31	low DNA	pos	*****	73	low DNA	neg	***
32	low DNA	pos	*****	74	low DNA	faint pos	***
33	low DNA	pos	****	75	Low DNA	pos	***
34	low DNA	faint pos	*****	76	Low DNA	pos	neg
35	low DNA	pos	****	77	low DNA	faint pos	*****
36	low DNA	pos	neg	78	low DNA	pos	*****
37	low DNA	pos	****	79	1	pos	**
38	low DNA	neg	*****	80	low DNA	pos	****
39	1	pos	****	81	low DNA	pos	****
40	low DNA	pos	*	82	low DNA	pos	****
41	low DNA	pos	*	83	low DNA	neg	*****
42	low DNA	pos	**	84	low DNA	pos	****
				85	low DNA	pos	**
				86	low DNA	pos	*
standard	AAD1	5ug/ml	*****				
standard	AAD1	0.5ug/ml	**				

[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 transformation of rye, *see, e.g.*, Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.*, Hinchey *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), H₂O 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 (vI)* for internal consistency.

[00438] 17.2 – Completion of *AAD-2 (vI)* binary vector.

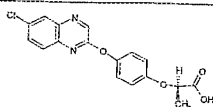
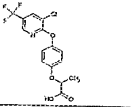
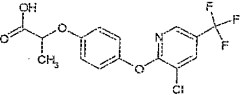
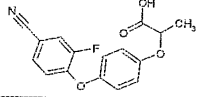
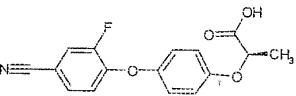
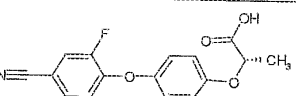
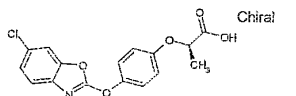
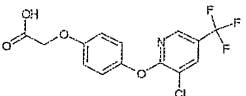
[00439] The *AAD-2 (vI)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (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 OSM 3'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 OSM 5'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 μ M Fe²⁺, 200 μ M Na ascorbate, 1 mM α -ketoglutarate using 4 μ l AAD1 (32 μ g protein) extract or 3 μ l AAD2 extract (42 μ g protein).

STRUCTURE	Reg ID	Compound	Enantiomer	A510	
				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 μ M 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 K_m and “ V_{max} ” values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	K_m (μM) $\pm SE$	V_{max} (A510 units)	K_m/V_{max} (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 Fe^{2+} 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-leaf 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 ($22\pm 5^{\circ}\text{C}$, $50\pm 30\%$ RH, 14 hours light:10 dark, minimum $500\ \mu\text{E}/\text{m}^2\text{s}^{-1}$ 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* T₁ 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 T₁ *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 T₁ plants survived 200 g ae/ha 2,4-D uninjured (population injury average = 34%), and >73% (11 of 15) *AAD-1* (v3) T₁ 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 T₁ 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 Agrilience 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 Agrilience 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 art 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* ($\nu 3$)-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* ($\nu 3$) 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* ($\nu 3$) 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 Agrilience (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1* ($\nu 3$), 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* ($\nu 3$) 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* ($\nu 3$) 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* ($\nu 3$)-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 Agrilience 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 art 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* ($\nu 3$) 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* ($\nu 3$) 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 Agrilience (2003). Each alternative herbicide enabled for use in HTC's by *AAD-1* ($\nu 3$), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-1* ($\nu 3$) 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* ($\nu 3$) 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* ($\nu 3$), 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* ($\nu 3$) 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* ($\nu 3$) + 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 Agrilience (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 (Agrilience, 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 Agrilience (2003). Each alternative herbicide enabled for use in HTC 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 (Agrilience, 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 Agrilience (2003). Each alternative herbicide enabled for use in HTC 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 Phytigel (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 sprayed 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