8357938

THE UNITED STATES OF AMERICA

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UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 21, 2023

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APPLICATION NUMBER: ***11/587,893***
FILING DATE: ***May 22, 2008***
PATENT NUMBER: ***7838733***
ISSUE DATE: ***November 23, 2010***

UNITED STATES PATENT AND TRADEMARK OFFICE · DEPARTMENT OF COMMERCE

Certified by

Kathi

Performing the Functions and Duties of the
Under Secretary of Commerce
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IAP6 Rec'd PCT/PTO 27 OCT 2006

PTO-1390 (Rev. 07-2005)
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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER: DAS-115XC1 U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 11/587893
INTERNATIONAL APPLICATION NO.: PCT/US2005/014737	INTERNATIONAL FILING DATE: May 2, 2005	PRIORITY DATE CLAIMED: April 30, 2004
TITLE OF INVENTION: Novel Herbicide Resistance Genes		
APPLICANT(S) FOR DO/EO/US: Terry Wright, Justin Lira, Donald Merlo, Nicole Hopkins		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a submission under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a submission under 35 U.S.C. 371.
3. ☑ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☑ The US has been elected (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☑ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☑ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☑ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☑ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). unsigned
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A preliminary amendment.
14. ☐ An Application Data Sheet under 37 CFR 1.76.
15. ☐ A substitute specification.
16. ☐ A power of attorney and/or change of address letter.
17. ☑ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.2 and 37 CFR 1.821- 1.825.
18. ☐ A second copy of the published International Application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

This collection of information is required by 37 CFR 1.414 and 1.491-1.492. 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 15 minutes to complete, including gathering information, preparing, and submitting the completed 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: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
11/587893	PCT/US2005/014737	DAS-115XC1

20. Other items or information:

Copy of cover page of the published PCT application; Certificate of Express Mailing; Statement Regarding Sequence Listing; Assignee Information; Return Receipt Postcard

The following fees have been submitted	CALCULATIONS	PTO USE ONLY
21. ☑ Basic national fee (37 CFR 1.492(a)) $300	$ 300.00	
22. ☑ Examination fee (37 CFR 1.492(c)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) .. .. $0 All other situations .. $200	$ 0.00	
23. ☑ Search fee (37 CFR 1.492(b)) If the written opinion of the ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) .... $0 Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority .. ... . $100 International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB .. .. $400 All other situations $500	$ 0.00	
TOTAL OF 21, 22 and 23 =	$300.00	
☐ Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) or computer program listing in an electronic medium) (37 CFR 1.492(j)). The fee is $250 for each additional 50 sheets of paper or fraction thereof.		

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof (round up to a whole number)	RATE	
215 - 100 =	115 /50 =	3	x $250	$750.00

Surcharge of $130.00 for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	67 - 20 =	47	x $50	$2,350.00	
Independent claims	5 - 3 =	2	x $200	$400.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $360	$ 0.00	
			TOTAL OF ABOVE CALCULATIONS =	$3,800.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. Fees above are reduced by ½.				$0.00	
			SUBTOTAL =	$3,800.00	
Processing fee of $130.00 for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i)). +				$	
			TOTAL NATIONAL FEE =	$3,800.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +				$0.00	
			TOTAL FEES ENCLOSED =	$ 3,800.00	
			Amount to be refunded:	$	
			Amount to be charged	$	

Bayer EX1005-0003 (1 of 2)

PTO-1390 (Rev. 07-2005)
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a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☑ Please charge my Deposit Account No. 19-0065 in the amount of $ 3,800.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0065. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the International Application to pending status.

SEND ALL CORRESPONDENCE TO:

CUSTOMER NUMBER
23557

October 27, 2006
Date

SIGNATURE
Jay M. Sanders
NAME
39,355
REGISTRATION NUMBER

Bayer EX1005-0004 (1 of 2)

11/587893
IAP6 Rec'd PCT/PTO 27 OCT 2006

Patent Application
Docket No. DAS-115XC1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry Wright, Justin Lira, Donald Merlo, Nicole Hopkins
Docket No.	:	DAS-115XC1
For	:	Novel Herbicide Resistance Genes

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

CERTIFICATE OF MAILING BY EXPRESS MAIL (37 CFR §1.10)

Express Mail No. EV397088068US Date of Deposit: October 27, 2006

I hereby certify that the attached Transmittal Letter to the United States Designated/Elected Office (DO/EO/US), cover page of published PCT application, unexecuted Declaration and Power of Attorney, Preliminary Amendment, computer-readable form of the sequence listing (disk), Statement Regarding Sequence Listing, and Application Data Sheet, with copies as required for authorization for use of Deposit Account No. 19-0065, are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date indicated above and are addressed to: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313.

David M. Gilland
Name of person mailing paper

Signature

J:\DAS\DAS-115XC1 US\PTO\certmail.doc/DNB/ehm

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 November 2005 (17.11.2005)

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(10) International Publication Number
WO 2005/107437 A2

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(21) International Application Number: PCT/US2005/014737

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(30) Priority Data:
60/567,052 30 April 2004 (30.04.2004) US

(71) Applicant *(for all designated States except US)*: **DOW AGROSCIENCES LLC** [US/US]; 9330 Zionsville Road, Indianapolis, IN 45268-1054 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WRIGHT, Terry, R.** [US/US]; 14162 Charity Chase Circle, Westfield, IN 46074 (US). **LIRA, Justin, M.** [US/US]; 12373 Ensley Drive, Fishers, IN 46038 (US). **MERLO, Donald, J.** [US/US]; 11845 Durbin Drive, Carmel, IN 46032 (US). **HOPKINS, Nicole** [US/US]; 2518 Chaseway Ct., Indianapolis, IN 46268 (US).

(74) Agents: **SANDERS, Jay, M.** et al.; Saliwanchik, Lloyd & Saliwanchik, A Professional Association, P.O. Box 142950, Gainesville, FL 32614-2950 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2005/107437 A2

(54) Title: NOVEL HERBICIDE RESISTANCE GENES

(57) Abstract: The subject invention provides novel plants that are not only resistant to 2,4-D and other phenoxy auxin herbicides, but also to aryloxyphenoxypropionate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention alone or "stacked" together with another herbicide resistance gene, preferably a glyphosate. resistance gene, so as to provide broader and more robust weed control, increased treatment flexibility, and improved herbicide resistance management options. More specifically, preferred enzymes and genes for use according to the subject invention are referred to herein as *AAD* (aryloxyalkanoate dioxygenase) genes and proteins. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. This highly novel discovery is the basis of significant herbicide tolerant crop trait opportunities as well as development of selectable marker technology. The subject invention also includes related methods of controlling weeds. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the formation of, and controlling, weeds that are resistant (or naturally more tolerant) to one or more herbicides such as glyphosate.

COPY

Bayer EX1005-0006 (1 of 2)

PTO/SB/01A (09-04)
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	NOVEL HERBICIDE RESISTANCE GENES

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☑ Application No. PCT/US2005/014737, filed on MAY 2, 2005,

☐ as amended on _____(if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be 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.

All statements made herein of my/own knowledge are true, all statements made herein 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 are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: TERRY WRIGHT

Signature: _____ Citizen of: US

Inventor two: JUSTIN LIRA

Signature: _____ Citizen of: US

Inventor three: DONALD MERLO

Signature: _____ Citizen of: US

Inventor four: NICOLE HOPKINS

Signature: _____ Citizen of: US

☐ Additional inventors or a legal representative are being named on _____ additional form(s) attached hereto.

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 1 minute 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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POWER OF ATTORNEY and CORRESPONDENCE ADDRESS INDICATION FORM

Application Number	
Filing Date	October 27, 2006
First Named Inventor	Terry Wright
Title	Novel Herbicide Resistance Genes
Art Unit	
Examiner Name	
Attorney Docket Number	DAS-115XC1

I hereby revoke all previous powers of attorney given in the above-identified application.

I hereby appoint:

☑ Practitioners associated with the Customer Number: 23557

OR

☐ Practitioner(s) named below:

Name	Registration Number

as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.

Please recognize or change the correspondence address for the above-identified application to:

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

I am the:

☑ Applicant/Inventor.

☐ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

SIGNATURE of Applicant or Assignee of Record

Signature		Date	
Name	NICOLE HOPKINS	Telephone	
Title and Company			

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 4 forms are submitted.

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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OR

☐ The address associated with Customer Number:

OR

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

I am the:

☑ Applicant/Inventor.

☐ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

SIGNATURE of Applicant or Assignee of Record

Signature		Date	
Name	TERRY WRIGHT	Telephone	
Title and Company			

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 4 forms are submitted.

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OR

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OR

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

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☑ Applicant/Inventor.

☐ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

SIGNATURE of Applicant or Assignee of Record

Signature		Date	
Name	DONALD MERLO	Telephone	
Title and Company			

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 4 forms are submitted.

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☐ Practitioner(s) named below:

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as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.

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☑ The address associated with the above-mentioned Customer Number:

OR

☐ The address associated with Customer Number:

OR

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

I am the:

☑ Applicant/Inventor.

☐ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

SIGNATURE of Applicant or Assignee of Record

Signature		Date	
Name	JUSTIN LIRA	Telephone	
Title and Company			

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 4 forms are submitted.

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

11/02/2006 TLUU11 00000099 190065 11587893

01 FC:1631	300.00	DA
02 FC:1681	750.00	DA
03 FC:1615	2350.00	DA
04 FC:1614	400.00	DA

PTO-1556
(5/87)

U.S. Government Printing Office: 2002 — 460-267/60233

11/587893
IAP6 Rec'd PCT/PTO 27 OCT 2006

PRELIMINARY AMENDMENT
Patent Application
Docket No. DAS-115XC1

October 27, 2006

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Terry Wright, Justin Lira, Donald Merlo, Nicole Hopkins

Docket No. : DAS-115XC1

For : Novel Herbicide Resistance Genes

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

PRELIMINARY AMENDMENT

Sir:

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

In the Specification

Please amend paragraph *[0001]* as follows:

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is the U.S. national stage application of International Patent Application No. PCT/US2005/014737, filed May 2, 2005, which claims ~~priority to~~ the benefit of U.S. provisional application Serial No. 60/567,052, filed April 30, 2004, the disclosures of which are hereby incorporated by reference in their entirety, including all figures, tables and amino acid or nucleic acid sequences.

After page 168: Please insert as new page 169 the attached Abstract of the Disclosure.

After new page 169: Beginning on a new page, please insert the attached substitute Sequence Listing.

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In the Claims

1 (original). A transgenic plant cell comprising a polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO:9 or a variant of SEQ ID NO:9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO:9.

2 (original). The cell of claim 1 wherein said plant cell is selected from the group consisting of dicotyledonous cells and monocotyledonous cells.

3 (original). The cell of claim 2 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

4 (original). The cell of claim 2 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

5 (original). A transgenic plant comprising a plurality of cells according to claim 1, wherein expression of said polynucleotide renders said cells tolerant to an aryloxyalkanoate herbicide.

6 (original). The plant of claim 5 wherein said herbicide is a phenoxy auxinic herbicide.

7 (original). The plant of claim 5 wherein said herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

8 (original). The plant of claim 5 wherein said herbicide is an aryloxyphenoxypropionate.

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9 (original). The plant of claim 5 wherein said herbicide is selected from the group consisting of fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

10 (original). The plant of claim 5 wherein expression of said polynucleotide renders said plant resistant to both a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

11 (original). The plant of claim 5 wherein said plant further comprises a second herbicide resistance gene.

12 (original). The plant of claim 11 wherein said second herbicide resistance gene renders said plant resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), and inhibitors of protoporphyrinogen oxidase (PPO).

13 (original). A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 5, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

14 (original). The method of claim 13 wherein said herbicide is an R-enantiomer of a chiral phenoxy auxin.

15 (original). The method of claim 14 wherein said herbicide is selected from the group consisting of R-dichlorprop and R-mecoprop.

16 (original). The method of claim 13 wherein said herbicide is an achiral phenoxy auxin.

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17 (original). The method of claim 16 wherein said herbicide is selected from the group consisting of 2,4-D and MCPA.

18 (original). The method of claim 13 wherein said first herbicide is an aryloxyphenoxypropionate and said plant is a monocot.

19 (original). The method of claim 18 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, sorghum, and pasture grasses.

20 (original). The method of claim 13 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

21 (original). The method of claim 20 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

22 (original). The method of claim 13 wherein said method comprises applying a second herbicide.

23 (original). The method of claim 22 wherein said first herbicide and said second herbicide are applied sequentially.

24 (original). The method of claim 22 wherein said first herbicide and said second herbicide are applied concurrently.

25 (original). The method of claim 22 wherein said first herbicide is a phenoxy auxin and second herbicide is an aryloxyphenoxypropionate.

26 (original). The method of claim 13 wherein said plant is resistant to glyphosate.

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27 (original). The method of claim 14 wherein said R-enantiomer is a component of a racemic mixture.

28 (original). The method of claim 22 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

29 (original). The method of claim 28 wherein said second gene is selected from the group consisting of a modified AHAS (acetohydroxyacid synthase), SurA, SurB, Csr1, Csr1-1, Csr1-2, a modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT, PAT (phosphinothricin-N-acetyltransferase), bar, and a dicamba-degrading enzyme.

30 (original). The method of claim 28 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase inhibitors, protoporphyrinogen oxidase inhibitors, and hydroxyphenyl-pyruvate-dioxygenase inhibitors.

31 (original). The method of claim 30 wherein said second herbicide is an acetolactate synthase inhibitor selected from the group consisting of imidazolinones, sulfonyl ureas, and triazolopyrimidine herbicides.

32 (original). The method of claim 31 wherein said second herbicide is an imidazolinone selected from the group consisting of imazamox, imazethapyr, imazaquin, and imazapic.

33 (original). The method of claim 28 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

34 (original). The method of claim 33 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

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35 (original). The method of claim 28 wherein said first herbicide is an aryloxyphenoxypropionate and said second herbicide is glyphosate.

36 (original). The method of claim 35 wherein said first herbicide is selected from the group consisting of quizalofop, haloxyfop, and cyhalofop.

37 (original). The method of claim 31 wherein said second herbicide is a sulfonyl urea selected from the group consisting of amidosulfuron, bensulfuron, chlorimuron, chlorsulfuron, cinosulfuron, flupyrsulfuron, foramsulfuron, halosulfuron, nicosulfuron, primisulfuron, prosulfuron, rimsulfuron, sulfometuron, sulfosulfuron, thifensulfuron, triasulfuron, trifloxysulfuron, and triflusulfuron.

38 (original). The method of claim 28 wherein said method further comprises applying a third herbicide.

39 (original). The method of claim 38 wherein said herbicides are 2,4-D; quizalofop; and glufosinate.

40 (original). A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said first herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate.

41 (original). The method of claim 40 wherein said first herbicide is an acid, an inorganic salt, an organic salt, an ester, an R-enantiospecific isomer, or a component of a racemic mixture.

42 (original). The method of claim 40 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

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43 (original). The method of claim 42 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate

44 (original). A polynucleotide optimized for expression in a plant wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

45 (original). The polynucleotide of claim 44 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

46 (original). The polynucleotide of claim 44 wherein said polynucleotide comprises SEQ ID NO:5.

47 (original). An isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell.

48 (original). The polynucleotide of claim 47 wherein said promoter is a plant promoter.

49 (original). The polynucleotide of claim 47 wherein said promoter is a cassava vein mosaic virus promoter.

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50 (currently amended). A method of selecting for a transformed plant cell wherein said method comprises subject a plurality of plant cells to transformation with a polynucleotide of claim [[44 or]] 47 and growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxyalkanoate.

51 (original). The method of claim 50 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

52 (canceled).

53 (currently amended). A method of detecting whether a plant comprises a polynucleotide of claim 47 [[or 50]] wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

54 (original). The method of claim 53 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

55 (original). The method of 53 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

56 (original). The method of 54 wherein said method comprises using an antibody to detect for the presence of said protein.

57 (original). A seed comprising a plant cell of claim 1.

58 (original). A plant grown from the seed of claim 57.

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59 (original). A part, progeny, or asexual propagate of the plant of claim 5.

60 (original). The method of claim 13 wherein said method is used to treat or prevent herbicide-resistant weeds.

61 (original). The plant of claim 5 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus*, and *Xenorhabdus*.

62 (original). The plant of claim 5 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

63 (original). A method of conferring 2,4-D herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a nucleic-acid-encoding enzyme having R-specific aryloxyalkanoate dioxygenase activity relative to a 2,4-D herbicide.

64 (original). A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 47, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

65 (original). The method of claim 64 wherein said herbicide is a phenoxy auxin.

66 (original). The method of claim 64 wherein said herbicide is an aryloxy phenoxypropionate.

67 (original). The method of claim 64 wherein said plants are dicots.

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68 (original). The method of claim 64 wherein said plants are monocots.

Bayer EX1005-0023 (1 of 2)

Remarks

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

Respectfully submitted,

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JMS/ehm
Attachments: Abstract of the Disclosure
Substitute Sequence Listing

Bayer EX1005-0024 (1 of 2)

ABSTRACT

[00520] 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.

Bayer EX1005-0025 (1 of 2)

11/587895

APPLICATION DATA SHEET

IAP6 Rec'd PCT/PTO 27 OCT 2006

Application Information

Application Type::	Regular (National Stage)
Subject Matter::	Utility
Suggested Classification::	None
Suggested Group Art Unit::	None
CD-ROM or CD-R?::	None
Number of CD disks::	None
Number of copies of CDs::	None
Sequence submission?::	Paper
Computer Readable Form?::	Yes
Number of Copies of CRF::	One
Title::	NOVEL HERBICIDE RESISTANCE GENES
Attorney Docket Number::	DAS-115XC1
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	None
Total Drawing Sheets::	24
Small Entity?::	No
Petition included?::	No
Petition Type::	N/A
Secrecy Order in Parent Appl.?::	No

Bayer EX1005-0026 (1 of 2)

Applicant Information

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Bayer EX1005-0027 (1 of 2)

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Bayer EX1005-0028 (1 of 2)

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Bayer EX1005-0029 (1 of 2)

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This application is a	National Stage of	PCT/US2005/014737	May 2, 2005
PCT/US2005/014737	An application claiming the benefit under 35 USC 119(e) of	60/567,052	April 30, 2004

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Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46268-1054

Bayer EX1005-0030 (1 of 2)

11/587893
IAP6 Rec'd PCT/PTO 27 OCT 2006

Patent Application
Docket No. DAS-115XC1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry Wright, Justin Lira, Donald Merlo, Nicole Hopkins
Docket No.	:	DAS-115XC1
For	:	Novel Herbicide Resistance Genes

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

STATEMENT UNDER 37 CFR §1.821

Sir:

Transmitted herewith is a preliminary amendment containing a substitute sequence listing under 37 CFR §§1.821 through 1.825.

The substitute sequence listing is submitted in computer readable form and on paper. I hereby certify that the attached sequence listing in computer readable form is identical to the attached paper copy of the sequence listing and that no new material is introduced by this submission.

Respectfully submitted,

Oct. 27, 2006
Date Signed

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JMS/ehm

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11/587893
IAP6 Rec'd PCT/PTO 27 OCT 2006

SEQUENCE LISTING

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Bayer EX1005-0032 (1 of 2)

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Bayer EX1005-0033 (1 of 2)

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<223> rdpA(ncoI) 5' primer

<400> 6
cccatggctg ctgcactgtc ccccctctcc 30

<210> 7
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> 3'saci primer

<400> 7
gagctcacta gcgcgccggg cgcacgccac cga 33

Bayer EX1005-0035 (1 of 2)

<210> 8
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> BstEII/ Del NotI 5' primer

<400> 8
tggtggtgac ccatccgggc agcggctgca agggcc 36

<210> 9
<211> 295
<212> PRT
<213> Sphingobium herbicidovorans

<400> 9

Met His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1 5 10 15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
20 25 30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
35 40 45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
50 55 60

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

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
85 90 95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
100 105 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
115 120 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
130 135 140

Bayer EX1005-0036 (1 of 2)

```
Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295


<210>  10
<211>  295
<212>  PRT
<213>  Artificial Sequence

<220>
<223>  AAD-1 v2 Translation


<220>
<221>  MISC_FEATURE
<222>  (2)..(2)
<223>  Different from v1

<220>
<221>  MISC_FEATURE
```


Bayer EX1005-0037 (1 of 2)

<222> (212)..(212)
<223> Different from v1

<400> 10

```
Met Ala Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1               5                   10                  15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
            20                  25                  30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60

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

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205
```


Bayer EX1005-0038 (1 of 2)

```
Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295
```

<210> 11
<211> 296
<212> PRT
<213> Artificial Sequence

<220>
<223> AAD-1 v3 Translation

<220>
<221> MISC_FEATURE
<222> (2)..(3)
<223> Different from V1

<400> 11

```
Met Ala His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile
1               5                   10                  15

Ala Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val
            20                  25                  30

Asp Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp
        35                  40                  45

Ala Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr
    50                  55                  60
```


Bayer EX1005-0039 (1 of 2)

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

Val Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile
                85                  90                  95

Arg Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His
            100                 105                 110

Thr Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg
        115                 120                 125

Ala Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met
    130                 135                 140

Tyr Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu
145                 150                 155                 160

Gly Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr
                165                 170                 175

Gln Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp
            180                 185                 190

Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
        195                 200                 205

Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
    210                 215                 220

Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
225                 230                 235                 240

Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
                245                 250                 255

Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
            260                 265                 270

Ala Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr
        275                 280                 285
```


Bayer EX1005-0040 (1 of 2)

Val Gly Gly Val Arg Pro Ala Arg
290 295

<210> 12
<211> 888
<212> DNA
<213> Bradyrhizobium japonicum USDA 110

<400> 12
atgacgatcg ccatccggca gcttcagacg cattttgtcg gccaggtttc cggcctcgat 60

ttgcgaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac 120

gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg 180

aacttcggcc agcgcgagga cgcgcgcggc ggcacggtca ccaaggagaa ggactaccgg 240

ctgcaatccg gcctgaacga cgtctccaat ctcggcaagg acggcaagcc gctggccaag 300

gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc 360

cgtcccattc ccgcaaaatt ctcgctgctg tcggcgcgcg tggtgaaccc gacgggcggc 420

aacaccgaat tcgcggacat gcgcgccgcc tatgacgcgc tcgacgacga gaccaaggcc 480

gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc 540

accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc 600

acccatccgg tccaccgccg caagtcgctg tatctctcgt cgcatgccgg caagatcgcc 660

agcatgagcg tgccggaggg gcggctgctg ttgcgcgatc tcaacgagca cgcgacgcag 720

ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc 780

cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgccgcgcg 840

acggtggcgg ggacggagcc gacggtgcag cagcaggcgg cggagtag 888

<210> 13
<211> 289
<212> PRT
<213> Bradyrhizobium japonicum USDA 110

<400> 13

Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
1 5 10 15

Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
20 25 30

Bayer EX1005-0041 (1 of 2)

```
Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
        35                  40                  45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
    50                  55                  60

Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
        115                 120                 125

Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
    130                 135                 140

Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
145                 150                 155                 160

Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
                165                 170                 175

Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
        195                 200                 205

Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
    210                 215                 220

Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240

Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
                245                 250                 255
```


Bayer EX1005-0042 (1 of 2)

Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
260 265 270

Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
275 280 285

Val

<210> 14
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> brjap 5'(speI)

<400> 14
actagtaaca aagaaggaga tataccatga cgat 34

<210> 15
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> br jap 3' (xhoI)

<400> 15
ttctcgagct atcactccgc cgcctgctgc tgc 33

<210> 16
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 forward primer

<400> 16
gtaaaacgac ggccagt 17

<210> 17
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 reverse primer

Bayer EX1005-0043 (1 of 2)

<400> 17
caggaaacag ctatgac 17

<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> NcoI of Brady

<400> 18
tataccacat gtcgatcgcc atccggcagc tt 32

<210> 19
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> SacI of Brady

<400> 19
gagctcctat cactccgccg cctgctgctg cac 33

<210> 20
<211> 525
<212> PRT
<213> Glycine max

<400> 20

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1 5 10 15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
20 25 30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
35 40 45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
50 55 60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65 70 75 80

Bayer EX1005-0044 (1 of 2)

```
Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
                85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300
```


Bayer EX1005-0045 (1 of 2)

```
Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525
```


Bayer EX1005-0046 (1 of 2)

<210> 21
<211> 525
<212> PRT
<213> Artificial sequence

<220>
<223> Doubly mutated Soybean EPSPS protein: threonine 183 converted to isoleucine; proline 187 converted to serine

<400> 21

```
Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1               5                  10                  15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
            20                  25                  30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65                  70                  75                  80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
                85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175
```


Bayer EX1005-0047 (1 of 2)

```
Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400
```


Bayer EX1005-0048 (1 of 2)

```
Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525
```

<210> 22
<211> 1604
<212> DNA
<213> Artificial sequence

<220>
<223> Soybean-biased DNA sequence encoding doubly-mutated EPSPS disclosed in SEQ ID NO:21, with added sequence

<220>
<221> misc_feature
<222> (1)..(1575)
<223> Coding sequence of soybean-biased DNA sequence encoding the EPSPS protein of SEQ ID NO:21

<220>
<221> misc_feature
<222> (1576)..(1578)
<223> Translation terminator tga

<220>
<221> misc_feature
<222> (1579)..(1604)
<223> Sequence included to introduce translation stop codons in all six

Bayer EX1005-0049 (1 of 2)

open reading frames, and to introduce a SacI restriction enzyme recognition site for cloning purposes

<400> 22
atggctcaag tctcccgtgt tcacaatctt gctcagtcaa cccaaatctt tggacattca 60

agcaactcaa acaaactgaa gtctgtgaat tctgtctcac ttcgcccacg cctttgggga 120

gcatccaaga gtcgcatacc aatgcacaag aatgggagtt tcatgggcaa cttcaatgtt 180

gggaaaggca attctggtgt cttcaaagtt tcagcttctg ttgcagccgc agagaaaccc 240

agcacttccc ctgagattgt tcttgaaccc attaaggact tcagtggaac aatcactctg 300

cctggatcaa agagtctttc aaacagaata cttctcttgg cagctctgag tgaaggaacc 360

actgtagttg acaacctttt gtactctgaa gatattcatt acatgttggg tgctctcaga 420

actcttgggt tgagagttga agatgacaag accacaaaac aagccatagt tgaaggatgt 480

ggtgggttgt ttccaacaag caaagaatcc aaagatgaga tcaacttgtt tcttggcaat 540

gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt 600

tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc 660

ctgaaacagc ttggagcaga tgttgattgc ttcttgggca caaactgccc tccagtgaga 720

gtgaatggga agggaggttt gcctggtgga aaggtcaaac tgagtggatc agtctcttcc 780

cagtatctga ctgccttgct catggctgcc cctctggctt tgggtgatgt ggagattgaa 840

atagtggaca agttgatttc tgttccatat gtggaaatga ccctcaaact catggagagg 900

tttggagttt ctgttgaaca ttctggcaac tgggatcgtt tccttgtaca tggaggtcag 960

aagtacaaaa gccctggcaa tgcctttgtt gaaggggatg caagctctgc ttcctatctc 1020

ttggctgggg ctgccatcac tggtgggacc atcactgtga atggctgtgg cacctcatcc 1080

cttcaaggtg atgtaaagtt tgcagaggtc ttggagaaaa tgggtgccaa ggtcacctgg 1140

tctgagaaca gtgtaactgt gtctggacct cccagagact tcagtggcag aaaggttctc 1200

cgtggaattg atgtgaacat gaacaagatg ccagatgtgg ccatgaccct cgctgttgta 1260

gccctgtttg caaatggacc aactgcaatc cgtgatgttg cttcatggag ggtgaaggag 1320

acagagagga tgattgccat ttgcacagaa ctccgcaaac ttggtgcaac agttgaagag 1380

ggaccagatt actgtgtgat aaccccacct gagaagctca atgtgacagc cattgacacc 1440

tatgatgacc acagaatggc aatggctttc tcccttgctg cctgtggtga tgtgcctgtg 1500

actatcaaag accctgggtg cacaaggaag acatttccag actactttga agttttggag 1560

Bayer EX1005-0050 (1 of 2)

aggttgacaa agcactgagt agttagctta atcacctaga gctc 1604

<210> 23
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Primer Pat 5-3

<400> 23
agataccctt ggttggttgc 20

<210> 24
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Primer Pat 3-3

<400> 24
cagatggatc gtttggaagg 20

<210> 25
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> AAD-1 PTU forward primer

<400> 25
ataatgccag cctgttaaac gcc 23

<210> 26
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> AAD-1 PTU reverse primer

<400> 26
ctcaagcata tgaatgacct cga 23

<210> 27
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Forward primer for Coding Region PCR AAD-1 (RdpAcodF)

Bayer EX1005-0051 (1 of 2)

<400> 27
atggctcatg ctgccctcag cc 22

<210> 28
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Reverse primer for Coding Region PCR AAD-1 (RdpAcodR)

<400> 28
cgggcaggcc taactccacc aa 22

<210> 29
<211> 932
<212> DNA
<213> Artificial sequence

<220>
<223> AAD-2 v2 plant-optimized nucleotide

<400> 29
ccatggctac catagcaatc agacagctcc agacccactt tgtgggtcaa gtttctggat 60

tggacctcag aaagccactc actcctggag aagccagaga agttgaatca gctatggaca 120

agtacgcagt tcttgtcttc catgaccaag acatcacaga tgagcaacag atggcctttg 180

ccctcaactt tggtcagagg gaggatgcac gtggtggcac tgtcaccaaa gagaaggatt 240

accgtcttca gtctggcctc aatgatgttt ccaacttggg caaagatgga aagccacttg 300

ccaaggacag ccgcacccat ttgttcaacc ttggaaactg cttgtggcat tctgactcca 360

gcttcagacc aatcccagcc aagttcagcc tcctttctgc tcgtgttgtg aacccaactg 420

gtgggaacac tgagtttgct gacatgagag ctgcctatga tgctcttgac gatgaaacca 480

aagctgagat tgaggacctt gtgtgtgagc actctctcat gtactcaagg ggctcacttg 540

gcttcactga gtacacagat gaagagaagc aaatgttcaa gcccgtcttg cagcgcttgg 600

tccgcacaca ccctgtgcac cgtcgcaaat cactctacct ctccagccat gccggaaaga 660

ttgccagcat gtccgtccct gaagggaggc tccttttgag ggatttgaat gaacatgcta 720

ctcagcctga gttcgtctat gttcacaaat ggaagttgca tgatcttgtg atgtgggaca 780

ataggcaaac catgcacaga gtgaggagat atgaccagtc ccaacccaga gacatgcgcc 840

gtgcaacagt tgctgggacc gagcccacag tgcaacagca agcagcagag tgagtagtta 900

gcttaatcac ctagagctcg gtcaccagat ct 932

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<220>
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<400> 30

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Glu Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp
        35                  40                  45

Gln Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly
    50                  55                  60

Gln Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr
65                  70                  75                  80

Arg Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly
                85                  90                  95

Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
            100                 105                 110

Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
        115                 120                 125

Ser Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu
    130                 135                 140

Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
145                 150                 155                 160

Ala Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg
                165                 170                 175

Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
            180                 185                 190
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Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
195 200 205

Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
210 215 220

Val Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr
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Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
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Met Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln
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IAP6 Rec'd PCT/PTO 27 OCT 2006

PTO-1390 (Rev. 07-2005)
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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER: DAS-115XC1 U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 11/587893
INTERNATIONAL APPLICATION NO.: PCT/US2005/014737	INTERNATIONAL FILING DATE: May 2, 2005	PRIORITY DATE CLAIMED: April 30, 2004

TITLE OF INVENTION: Novel Herbicide Resistance Genes

APPLICANT(S) FOR DO/EO/US: Terry Wright, Justin Lira, Donald Merlo, Nicole Hopkins

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a submission under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a submission under 35 U.S.C. 371.
3. ☑ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☑ The US has been elected (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☑ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☑ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☑ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☑ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). unsigned
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A preliminary amendment.
14. ☐ An Application Data Sheet under 37 CFR 1.76.
15. ☐ A substitute specification.
16. ☐ A power of attorney and/or change of address letter.
17. ☑ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.2 and 37 CFR 1.821- 1.825.
18. ☐ A second copy of the published International Application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

This collection of information is required by 37 CFR 1.414 and 1.491-1.492. 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 15 minutes to complete, including gathering information, preparing, and submitting the completed 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: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
11/587893	PCT/US2005/014737	DAS-115XC1

20. Other items or information:

Copy of cover page of the published PCT application; Certificate of Express Mailing; Statement Regarding Sequence Listing; Assignee Information; Return Receipt Postcard

The following fees have been submitted	CALCULATIONS	PTO USE ONLY
21. ☑ Basic national fee (37 CFR 1.492(a)) $300	$ 300.00	
22. ☑ Examination fee (37 CFR 1.492(c)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) .. .. $0 All other situations .. $200	$ 0.00	
23. ☑ Search fee (37 CFR 1.492(b)) If the written opinion of the ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) .... $0 Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority .. ... . $100 International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB .. .. $400 All other situations $500	$ 0.00	
TOTAL OF 21, 22 and 23 =	$300.00	
☐ Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) or computer program listing in an electronic medium) (37 CFR 1.492(j)). The fee is $250 for each additional 50 sheets of paper or fraction thereof.		

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof (round up to a whole number)	RATE	
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CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	$
Total claims	67	- 20 =	47	x $50	$2,350.00
Independent claims	5	- 3 =	2	x $200	$400.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ $360	$ 0.00
				TOTAL OF ABOVE CALCULATIONS =	$3,800.00
☐ Applicant claims small entity status. See 37 CFR 1.27. Fees above are reduced by ½.					$0.00
				SUBTOTAL =	$3,800.00
Processing fee of $130.00 for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i)). +					$
				TOTAL NATIONAL FEE =	$3,800.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +					$0.00
				TOTAL FEES ENCLOSED =	$ 3,800.00
				Amount to be refunded:	$
				Amount to be charged	$

Bayer EX1005-0056 (1 of 2)

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39,355
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Bayer EX1005-0057 (1 of 2)

11/587893
IAP6 Rec'd PCT/PTO 27 OCT 2006
Patent Application
Docket No. DAS-115XC1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry Wright, Justin Lira, Donald Merlo, Nicole Hopkins
Docket No.	:	DAS-115XC1
For	:	Novel Herbicide Resistance Genes

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Commissioner for Patents
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Alexandria, VA 22313

CERTIFICATE OF MAILING BY EXPRESS MAIL (37 CFR §1.10)

Express Mail No. EV397088068US Date of Deposit: October 27, 2006

I hereby certify that the attached Transmittal Letter to the United States Designated/Elected Office (DO/EO/US), cover page of published PCT application, unexecuted Declaration and Power of Attorney, Preliminary Amendment, computer-readable form of the sequence listing (disk), Statement Regarding Sequence Listing, and Application Data Sheet, with copies as required for authorization for use of Deposit Account No. 19-0065, are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date indicated above and are addressed to: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313.

David M. Gilland
Name of person mailing paper

Signature

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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 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 GENES

Cross-Reference to Related Application

[0001] This application claims priority to U.S. provisional application Serial No. 60/567,052, filed April 30, 2004.

Background of the Invention

[0002] Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

[0003] Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant corn, for example, can be sprayed to control weeds without significantly damaging the corn plants.

[0004] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (*e.g.,* wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (*e.g.*, alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; *see* "isb.vt.edu/cfdocs/fieldtests1.cfm , 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

[0005] Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has

been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

[0006] Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). (Ng *et al.*, 2003; Simarmata *et al.*, 2003; Lorraine-Colwill *et al.*, 2003; Sfiligoj, 2004; Miller *et al.*, 2003; Heap, 2005; Murphy *et al.*, 2002; Martin *et al.*, 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past 3-5 years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum, Lolium multiflorum, Eleusine indica, Ambrosia artemisiifolia, Conyza canadensis, Conyza bonariensis,* and *Plantago lanceolata.* Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

[0007] In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting

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no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agriliance, 2003).

[0008] 2,4-D is in the phenoxy acid class of herbicides, as are MCPA, mecoprop, and dichlorprop. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

[0009] 2,4-D has different levels of selectivity on certain plants (*e.g.,* dicots are more sensitive than grasses). Differential metabolism of 2,4-D by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

[0010] Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D is currently formulated to have a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

[0011] One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.* (1989), Lyon *et al.* (1989), Lyon (1993), and U.S. Patent No. 5,608,147).

[0012] A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many

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

[0013] One unique example with low homology to *tfdA* (28% amino acid identity) is *rdpA* from *Sphingobium herbicidovorans* (Kohler *et al.*, 1999, Westendorf *et al.*, 2002). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organisms that degrade phenoxypropionic acid were described some time ago, little progress had been made in characterizing this pathway until recently (Horvath *et al.*, 1990). An additional complication to dichlorprop degradation is the stereospecificity (R *vs.* S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes, or transformation of this gene into plants, has not heretofore been reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids (*e.g.*, 2,4-D).

[0014] Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

[0015] Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxy auxins (such as 2,4-D and dichlorprop), pyridyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. Discovery of a multifunctional enzyme for the degradation of herbicides covering multiple modes would be both unique and valuable as an HTC trait.

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Brief Summary of the Invention

[0016] The subject invention provides novel plants that are not only resistant to 2,4-D, but also to AOPP herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, imidazolinone-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

[0017] In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, AOPP, and one or more commercially available herbicides (*e.g.*, glyphosate, imidazolinones, glufosinate, sulfonylureas, dicamba, bromoxynil, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

[0018] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and AOPP herbicides, in an enantiospecific manner. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate

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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.

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[0024] **Figure 4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

[0025] **Figure 5** shows 2,4-dichlorophenol production by recombinant *AAD-1*.

[0026] **Figures 6A and 6B** show phenol production by recombinant *AAD-1* from various herbicide substrates.

[0027] **Figure 7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

[0028] **Figures 8A and 8B** show that *AAD-1 (v3)* was expressed equally in *Arabidopsis* leaves of different ages but continued to accumulate throughout the 25 days of experiment. Plants that were not sprayed with the herbicide 2,4-D (panel A) expressed a little more AAD-1 (v3) than those had been sprayed (panel B). Bars represent the mean ± SEM of 5 leaves from 5 different plants, with percent expression of AAD-1 (v3) normalized to total soluble protein. Light bars represent the third young leaves (N-3) collected from the top, dark bars represent the 5th oldest leaves from the bottom.

[0029] **Figures 9A, 9B, and 9C** show injury of *Arabidopsis* plants after 2,4-D treatment. Four different lines were each treated with four different doses of 2,4-D and their injury was graded 4 (panel A) and 14 (panel B) days after treatment. Their expression of AAD-1 (v3) in leaves was also determined using ELISA (panel C). The results were mean ± SEM of five leaves from five different plants received the same treatment.

[0030] **Figure 10** illustrates that pDAB3230-transformed *Arabidopsis* (*AAD-1* + *EPSPS*) shows >14-fold level of glyphosate tolerance 7 DAT vs. wildtype and transformed control *Arabidopsis* lines.

[0031] **Figure 11** shows dose response of callused maize suspensions to R-haloxyfop.

[0032] **Figure 12** shows that at 1 μM cyhalofop phenol, growth is still 76% as high as the control without cyhalofop phenol.

[0033] **Figure 13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

[0034] **Figure 14** shows the responses of several *AAD-1 (v3)*-transformed and non-transformed event clones to lethal doses of two AOPP herbicides (haloxyfop and quizalofop) applied as a postemergence spray 1 week prior.

[0035] **Figure 15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to

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quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

[0036] **Figure 16** shows *AAD-1 (v3)*-transformed corn tolerant to 8X field rates of quizalofop (Assure II) under field conditions.

[0037] **Figure 17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

[0038] **Figure 18** shows Western Blotting analysis on soybean calli transformed with *AAD-1 (V3)* gene indicating that the callus cells are expressing AAD-1 (v3) protein.

[0039] **Figure 19** shows fitted curves for 2,4-D degradation rates by AAD-2 (v1) vs. AAD-1 (v1).

[0040] **Figure 20** shows the response of *AAD-1 v3* (plant optimized), or *AAD-1 (v2)* (native), *AAD-2 (v1)* (native), or *AAD-2 (v2)* (plant optimized)-transformed T_1 *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T_1 family.

[0041] **Figure 21** shows western blot analysis of individual native *AAD-2 (v1)*-transformed T_1 *Arabidopsis* plants. This shows that plants expressing the AAD-2 (v1) protein are suffering severe injury from 200 g ae (acid equivalent)/ha 2,4-D treatments, which normally causes little injury to native *AAD-1 (v2)* or plant optimized *AAD-1 (v3)*-transformed *Arabidopsis*. AAD-2 protein is identified on the gel. Several background bands were detected in *AAD-2*-transformed and *Pat/Cry1F*-transformed samples.

[0042] **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop.

Brief Description of the Sequences

[0043] **SEQ ID NO:1** is the sequence of a forward primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0044] **SEQ ID NO:2** is the sequence of a reverse primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0045] **SEQ ID NO:3** is the nucleotide sequence of *AAD-1 (v1)* from *Sphingobium herbicidovorans*.

[0046] **SEQ ID NO:4** is the nucleic acid sequence of the native *AAD-1* gene with internal NotI restriction site removed. This gene is designated *AAD-1 (v2)*. DNA sequencing

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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

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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.

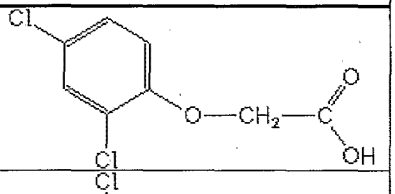
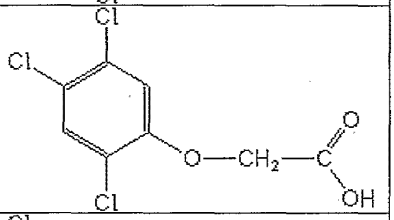
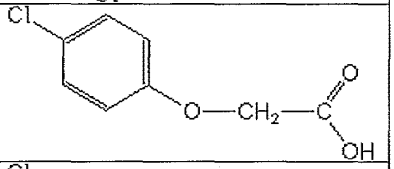
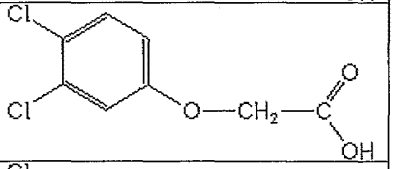
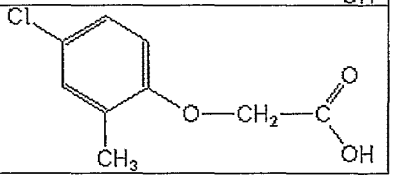
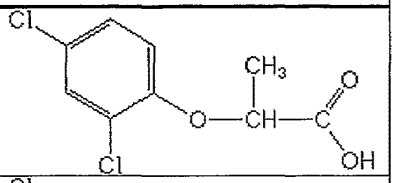
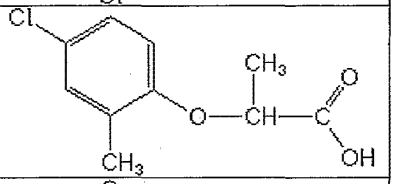
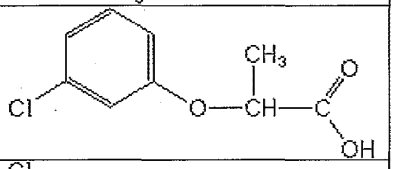
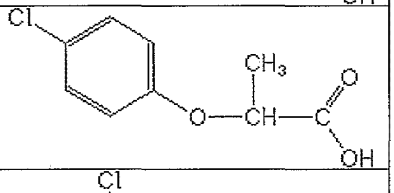
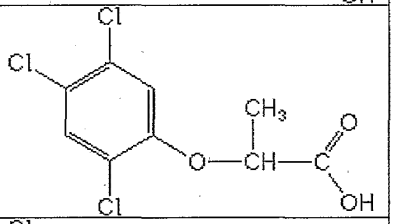
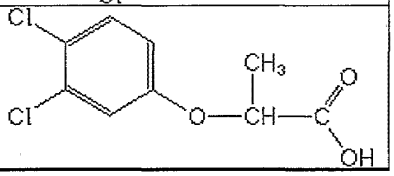
Chemical name	**CAS no**	**Possible use rate ranges (g ae/ha)**	**Preferred use rate ranges (g ae/ha)**	**Structure**
2,4-D	94-75-7	25 – 4000	280 - 1120	
2,4,5-T	93-76-5	25 – 4000	25 – 4000	
4-CPA	122-88-3	25 – 4000	25 – 4000	
3,4-DA	588-22-7	25 – 4000	25 – 4000	
MCPA	94-74-6	25 – 4000	125 - 1550	

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-

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season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

[0077] **Table 2.** AOPP graminicidal compounds listed by accepted common names. Reference to AOPP herbicides is generally made to the active acid but most are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-1 enzyme *in planta* as general plant esterases convert these esters to the active acids *in planta*. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. When chiral propionic acid, salt, or ester herbicides are indicated, racemic (R,S) or optically purified (R or S) enantiomers are considered the same herbicides for the purpose of naming these herbicides, even though different CAS numbers may correspond to optically pure compounds. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Table 2. AOPP graminicidal compounds listed by accepted common names.

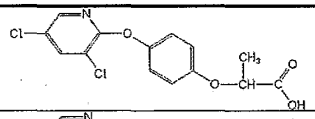
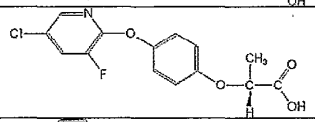
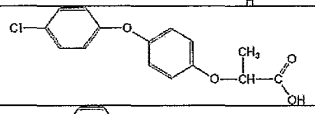
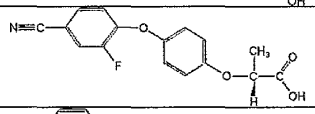
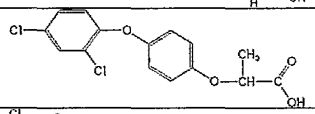
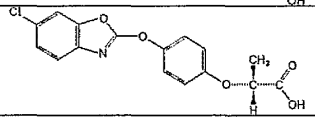
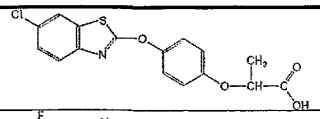
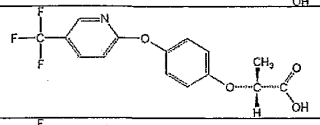
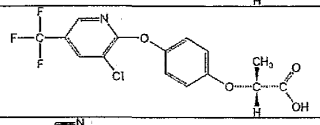
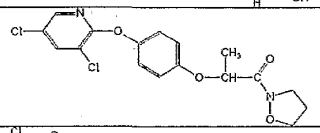
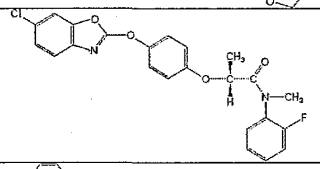
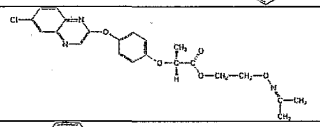
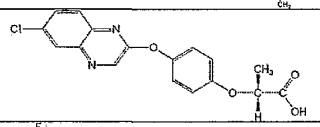
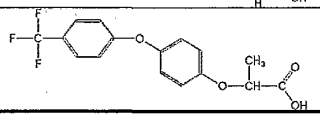
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	

Table 2. AOPP graminicidal compounds listed by accepted common names.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop	256412-89-2	10-2000	35-280	
Propaquizafop	111479-05-1	10-2000	30-240	
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

[0078] A single gene (*AAD-1*) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, *AAD-1* can provide protection *in planta* to AOPP herbicides where natural tolerance also was not sufficient to allow selectivity. Plants containing *AAD-1* alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a

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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

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environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

[0081] Herbicide formulations (*e.g.*, ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (*e.g.*, adjuvants or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

[0082] One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant species or resistant weed species could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (*e.g.*, resistant plant or bacterial *EPSPS, GOX, GAT*), glufosinate resistance (*e.g.*, *Pat, bar*), acetolactate synthase (ALS)-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrmidinylthiobenzoates, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD (4-hydroxlphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (*e.g.*, *PPO-1*), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), dicamba-degrading enzymes (*see, e.g.,* US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

[0083] Regarding additional herbicides, some additional preferred ALS inhibitors include the triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyrithiobac), and flucarbazone. Some preferred HPPD inhibitors include mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

[0084] Additionally, *AAD-1* alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (*e.g.*, insect resistance, fungal resistance, or stress

tolerance, *et al.*) or output (*e.g.*, increased yield, improved oil profile, improved fiber quality, *et al.*) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

[0085] The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. Even though this enzyme has very low homology to *tfdA*, the genes of the subject invention can still be generally classified in the same overall family of α-ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "$HX(D/E)X_{23-26}(T/S)X_{114-183}HX_{10-13}R$" motif which comprises the active site. The histidines coordinate Fe^{+2} ion in the active site that is essential for catalytic activity (Hogan *et al.*, 2000). The preliminary *in vitro* expression experiments discussed herein were tailored to help select for novel attributes.

[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.

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[0089] The subject invention also includes methods of controlling weeds wherein said methods comprise applying an AOPP herbicide and/or a phenoxy auxin herbicide to plants comprising an *AAD-1* gene.

[0090] In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also AOPP herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

[0091] Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

[0092] As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber *et al.*, 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. *See* U.S. Patent No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an α-ketoglutarate-dependent dioxygenase reaction (Smejkal *et al.*, 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (*e.g.*, cotton and tobacco) normally sensitive to 2,4-D (Streber *et al.*, 1989; Lyon *et al.*, 1989; Lyon *et al.*, 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of α-ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

[0093] *RdpA*, from *Sphingobium herbicidovorans* (Westendorf *et al.*, 2002), is one unique example with low homology (28% amino acid identity). This enzyme has been shown to catalyze the first step in (R)-dichlorprop (and other (R)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf *et al.*, 2003). Although the organism responsible for phenoxypropionic acid degradation has been known for some time, little progress has been made in characterizing this pathway until recently (Horvath *et al.*,

1990). An additional complication to dichlorprop degradation is the stereospecificity (R *vs.* S) involved in both the uptake (Kohler, 1999) and initial oxidation of dichlorprop (Westendorf *et al.*, 2003). Heterologous expression of *rdpA* in other microbes or transformation of this gene into plants, heretofore, was not reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids. There was no prior expectation that *rdpA* or *AAD-1* genes could be successfully expressed in plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

[0094] As described in more detail in the Examples below, *rdpA* was cloned from *Sphingobium herbicidovorans* and tested for substrate promiscuity among various herbicide chemical classes. This α-ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and dichlorprop (Westendorf *et al.*, 2002 and 2003). However, no α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. *RdpA* has never been expressed in plants, nor was there any motivation to do so because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

[0095] In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-1 proteins and genes. AAD-1 was presently confirmed to degrade a variety of phenoxyacetic and phenoxypropionic auxin herbicides *in vitro*. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance are the aryloxyphenoxypropionate (AOPP) grass herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities.

[0096] The broad spectrum grass AOPP herbicides are reported herein to be excellent substrates for AAD-1 as well as 2,4-D, dichlorprop, and other phenoxy auxins. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxy auxins) and a range of broad spectrum, highly active grass herbicides (AOPPs).

[0097] Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyalkanoic auxin herbicides, and aryloxyphenoxy-

alkanoate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

[0098] The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf and grass weeds. *AAD-1* can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits (*e.g.*, glyphosate resistance, glufosinate resistance, imidazolinone resistance, bromoxynil resistance, *et al.*), and insect resistance traits (*Cry1F, Cry1Ab, Cry 34/45, et al.*) for example. Additionally, *AAD-1* can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

[0099] In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with *AAD-1*-containing constructs and have demonstrated high levels of resistance to both the phenoxy auxin and AOPP herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention. As shown below in Example 6, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

[00100] Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-1. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

[00101] HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

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[00102] Additionally, glyphosate tolerant crops grown worldwide are prevalent. Many times in rotation with other glyphosate tolerant crops, control of glyphosate-resistant volunteers may be difficult in rotational crops. Thus, the use of the subject transgenic traits, stacked or transformed individually into crops, provides a tool for controlling other HTC volunteer crops.

[00103] This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

[00104] The transgenic expression of the subject *AAD-1* genes is exemplified in, for example, *Arabidopsis*, corn (maize), tobacco, cotton, rice, soybean, and canola. However, the subject invention can be applied to any other desired types of plants. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be applied to multiple other grass and other broadleaf crops. Likewise, 2,4-D can be more positively utilized in grass crops where tolerance to 2,4-D is moderate, and increased tolerance via this trait would provide growers the opportunity to use 2,4-D at more efficacious rates and over a wider application timing without the risk of crop injury.

[00105] Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed (auxins) and grass weeds (AOPPs). This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-1 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

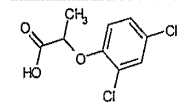
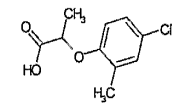
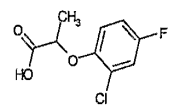
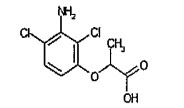
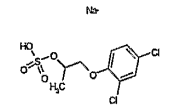
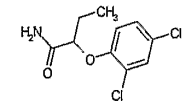
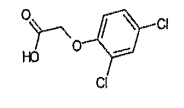
[00106] Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

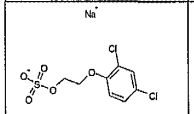
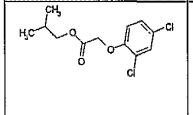
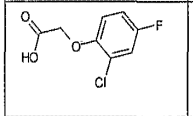
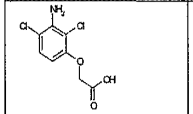
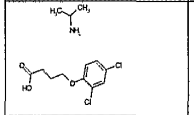
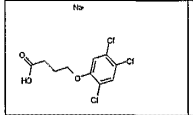
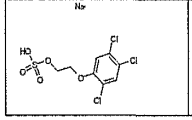
[00107] One gene of the subject invention, referred to herein as *AAD-1* (aryloxyalkanoate dioxygenase), was cloned from *Sphingobium herbicidovorans* (ATCC 700291) by PCR into pET 280-S/S (designated pDAB 3203) and expressed in BL-21 Star *E. coli.* When this gene is overexpressed (by induction of 1mM IPTG and culture lysate combined with the following reaction mix: 112.5 μg/ml 2,4-D, 1 mM Ascorbic acid, 1 mM α-ketoglutarate, 50 μM $Fe(NH_4)_2(SO_4)_2$, the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay). Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See **Tables 3 and 4**).

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[00108] **Table 3:** Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

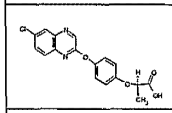
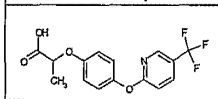
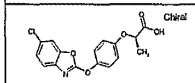
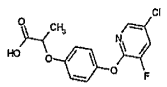
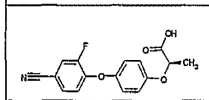
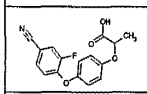
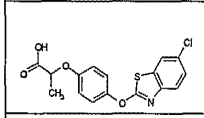
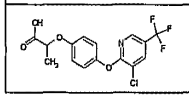
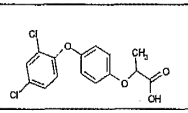
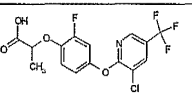
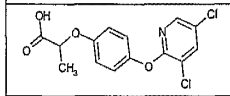
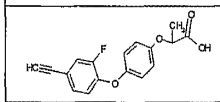
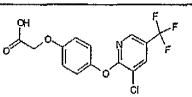
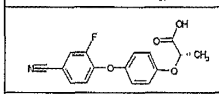
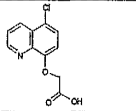
Table 3: Effect of purified AAD-1 (v1) on various herbicidal auxins and auxin analogs

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
	178587		0.003	0.02
	188527		0.003	0.036

[00109] **Table 4:** Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet. Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using either 1 μg or 10 μg (10X) purified AAD-1 (v1) per 0.16 ml assay.

Table 4. Effect of purified AAD-1 (v1) on various AOPP graminicides and analogs, and on cloquintocet.

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalofop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17
	11044492	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinofop	0.295	1.98
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63
	460511	(R,S)-diclofop	0.155	1.663
	25646		0.144	1.69
	70222	(R,S)-chlorazifop	0.128	1.584
	199608	cyhalofop	0.114	1.26
	43865	haloxyfop-oxyacetate	0.004	0.053
	7466	(S)-cyhalofop	0.003	0.017
	204558	Cloquintocet	0	0.001

[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

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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.*).

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[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.

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[00127] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis *et al.* 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz *et al.* 1983):

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

[00128] Washes can typically be carried out as follows:

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

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

[00129] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

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

(Suggs *et al.*, 1981).

[00130] Washes can typically be out as follows:

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

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

[00131] In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

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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

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

[00135] Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein. The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target pests and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

[00136] In addition, U.S. Patent No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

[00137] "Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus,

"focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

[00138] Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, or 297 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

[00139] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained

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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,

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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.

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[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.

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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.

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[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.

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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.

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Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00162] For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

[00163] In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

[00164] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

[00165] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.*, 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00166] In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, *see* U.S. Patent No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (*e.g.*, actin, ubiquitin, CaMV 35S, and the like). Tissue specific

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promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (*e.g.*, zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

[00167] Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

[00168] Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Patent No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Patent Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00169] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[00170] Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

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

[00171] As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence

already identified that encodes a protein with the desired characteristics (*i.e.*, α-ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case we chose only those sequences with ≤ 50% homology. We go on to exemplify that cloning and recombinantly expressing homologues with as little as 27% amino acid conservation can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

[00172] PCR and cloning of gene into pET. A single gene (*rdpA*) was identified from the NCBI database (*see* the ncbi.nlm.nih.gov website; accession #AF516752) as a homologue with only 28% amino acid identity to *tfdA* from *Ralstonia eutropha*. Percent identity was determined by first translating both the *rdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

[00173] The strain of *Sphingobium herbicidovorans* containing the *rdpA* gene was obtained from ATCC (American Type Culture Collection strain #700291). The lyophilized strain was revived according to ATCC protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 536. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days.

[00174] A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' TCT AGA AGG AGA TAT ACC ATG CAT GCT GCA CTG TCC CCC CTC TCC CAG CG 3' [(SEQ ID NO:1) (added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' CTC GAG TTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

[00175] Twenty microliter reactions were set up as follows: MasterMix 8 μl, ea. primer 1 μl (50 pmoles/μl), gDNA 2.5 μl, H_2O 7.5 μl. PCR was then carried out under the following

conditions: 94° C 45 sec, 52° C 1.5 minute, 72° C 1.5 minute, for 30 cycles, followed by a final cycle of 72° C 5 minute, using Eppendorf's Master Taq kit (Eppendorf cat. #0032 002.250). The resulting ~1kb PCR product was cloned into pCR 2.1 (Invitrogen cat. # K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00176] Ten of the resulting white colonies were picked into 4 ml Luria Broth + 50 μg/ml Kanamycin (LB K), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Promega Wizard Plus SV kit (Promega cat. #A1460) and following the included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward (5' GTA AAA CGA CGG CCA GT 3') (SEQ ID NO:16) and Reverse (5' CAG GAA ACA GCT ATG AC 3') (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein (SEQ ID NO:9) was given a new general designation for internal consistency *AAD-1 (v1)* (AryloxyAlkanoate Dioxygenase).

[00177] Using the restriction enzymes corresponding to the sites added with the primer linkers (Xba 1, Xho 1) *AAD-1 (v1)* was cut out of the pCR2.1 vector and ligated into a pET 280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth + 50 μg/ml Streptomycin & Spectinomycin (LB S/S) agar plates. To differentiate between *AAD-1 (v1)*:pET 280 and pCR2.1:pET 280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB S/S, and grown at 37° C for 4 hours with agitation.

[00178] Each culture was then spotted onto LB K plates, which were incubated at 37° C overnight. Colonies that grew on the LB K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before. This expression construct was given the designation pDAB 3203.

Example 2 – Expression and Testing

[00179] 2.1 – HPLC analysis.

[00180] Plasmid pDAB 3203 was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR 1878. For expression, plasmid DNA purified from TOP10F' culture using Promega's Wizard kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After

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transformation, 50 µl of the cells were plated onto LB S/S agar plates and incubated overnight at 37° C.

[00181] The next morning, all colonies from the entire plate were scraped into 100 mls LB in a 500 ml tri-baffled flask and incubated at 37° C/200 rpm for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C/200 rpm. All 100 ml of culture was centrifuged at 4000 rpm for 20 min. The supernatants were then discarded, and the pellets were resuspended in 10 ml of 50 mM MOPS. These were then subjected to three 45-sec rounds of sonication to lyse the cells. Following this, lysates were centrifuged at 15,000 rpm to remove cell debris. The supernatant was pipetted off and stored at 4° C. To check for recombinant expression, a 20 µl aliquot was run on a 4-20% Tris Glycine gel (Invitrogen cat. #EC60255).

[00182] After expression was confirmed, enzyme activity was tested as follows. First, an aliquot of the cell extract was desalted with a PD-10 cartridge (Amersham cat. #17-0435-01). This was then used for subsequent herbicide enzyme reactions.

[00183] For each reaction, the following were combined: 2,4-D (125 µg/ml), [Ascorbate (1 mM), Ferrous ion (50 µM), α-ketoglutarate (1 mM), in 100 mM MOPS], cell extract (100 µl). This reaction was then incubated at room temp for 30 min., after which the reaction was stopped with the addition of 0.1 N HCl until pH was between 2 and 3. Half of the reaction volume (~500 µl) was set aside for bioassay, the remaining volume was organically extracted using Solid Phase Extraction tubes (Fisher cat. #11-131-6), eluting with 400 µl of Acetonitrile + 0.05% TFA.

[00184] The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10µ C18(2) 250 x 4.6mm (Phenomenex cat. #00G-4253-E0), run at 50% ACN + 0.05% TFA: 50% H_2O + 0.05% TFA to 100% ACN + 0.05% TFA over 5 min.

[00185] 2.2 – Plate test bioassays for herbicide degradation.

[00186] Plant bioassays were used to determine if *in vitro* enzymatic herbicide transformation resulted in a concomitant loss in herbicidal activity. Because of the selective nature of the herbicides being tested (*i.e.*, monocot plants controlled by AOPP herbicides and dicot plants controlled by auxinic herbicides), wildtype *Agrostis palustris* var. Pencross and *Arabidopsis thaliana* var. Columbia were used as monocot and dicot test species, respectively. Each species is amenable to germination and growth in small Petri dishes.

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[00187] *Arabidopsis* seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 μL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @ 250 rpm). Bleach solution was decanted inside a sterile hood and rinsed three times with sterile water. Bentgrass seeds were surface sterilized for 20 minutes in a like manner.

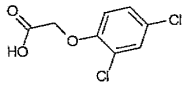
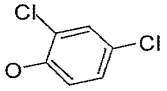
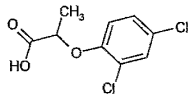
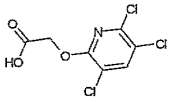
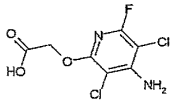
[00188] Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4 \cdot 2H_2O$, 10 μM NaCl, 10 nM $CoCl_2 \cdot H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60x15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

[00189] For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day^{-1}, 100 $\mu E/m^2s^1$, 23° C) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

[00190] This bioassay was used to confirm the loss of herbicidal activity as a result of AAD-1 (v1) degradation of the oxyalkanoate side chain from various agronomically relevant herbicides. In several instances, the anticipated phenol product co-eluted with the parent acid on HPLC and the bioassay served as the primary screen for herbicide degradation. **Tables 6 and 7** represent herbicidal substrates tested.

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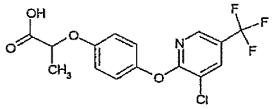
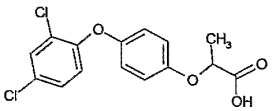
Table 6. *Arabidopsis* plate test bioassay for commercial phenoxy and pyridinyloxyalkanoate auxin substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
2,4-D	22	17	267	16	
DCP	>1000	nd	nd	nd	
Dichlorprop	nd	30	1000	33	
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

* Blank vector represents of cell lysate treatment where E. coli pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

Table 7. Bentgrass plate test bioassay for commercial aryloxyphenoxyalkanoate ACCase-inhibiting substrates.

Chemical tested	GR_{50} (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
Haloxyfop-RS	28	21	520	25	
Diclofop-RS	nd	20	130	7	

* Blank vector represents of cell lysate treatment where E. coli pET vector had no gene insert.

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vector treatments. A number ≥ 2 is considered the threshold for detecting herbicide activity loss with this assay.

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[00191] 2.3 – HPLC results.

[00192] From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see **Figure 1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme for being degraded. Based on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

[00193] Surprisingly, the initial HPLC results showed the presence of a new peak at 6.1 minute, in addition to a reduced 2,4-D peak at 5.5 min. This new peak was not present in the control assay. For an initial identification of the peak at 6.1 minutes, a DCP control was run under our assay conditions and predictably this also eluted at 6.1 minutes. The formation of this product was confirmed using a colorimetric assay to detect phenols (see example 3.1) as well as mass spectrometry. As expected, AAD-1 (v1) carries out a similar reaction as other members of this enzyme class. In the bioassay, these same samples were also shown to have an almost complete loss of 2,4-D herbicidal activity in the *Arabidopsis* plate assay (**Figure 2**). Regardless of the specific conditions of the assay (*i.e.*, longer incubations, more enzyme), only 50-75% of the 2,4-D could be degraded to DCP as measured by HPLC. In fact, longer induction of the BL-21 *E. coli* cells with IPTG only resulted in less active enzyme, even though more total recombinant protein was expressed.

[00194] After demonstrating degradation of 2,4-D, additional substrates were tested with similar ring substitutions (*i.e.*, oxyacetates and oxypropionates). The first compounds tested were the pyridine analogs fluroxypyr and triclopyr, which are pyridinyloxyacetates. No enzyme activity was detected on either of these as substrates. Additional tests on various analogs of these two pyridinyloxyacetates with either the fluorine or the amino groups removed also were not degraded. Interestingly however, adding a fluorine to the 5 position of 2,4-D resulted in an almost total loss of enzyme degradation (see next section for additional results).

[00195] ACCase inhibitors, haloxyfop and diclofop, were then tested using the same conditions as with 2,4-D. (The corresponding phenol metabolites co-eluted with the parent compound under the HPLC conditions used.) The bioassay results from these samples showed loss of herbicidal activity against both haloxyfop (**Figure 3**) and diclofop. These

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results were also confirmed by the colorimetric assay, which was also used to test a wider sampling of these compounds.

[00196] 2.4 – Plate test bioassays for herbicide degradation.

[00197] Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurified recombinant AAD-1 (v1) extracts (**Figure 2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR_{50} for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio of 2-3 typically correlated with 50-75% loss of parent herbicide activity (**Table 6**). Often a GR_{50} could not be determined following enzyme treatment; *de facto*, no detectable herbicide activity remained.

[00198] The AOPP class of herbicides, too, served as excellent substrates for AAD-1 (v1) as shown by near complete degradation of graminicidal activity using the bentgrass plate bioassay (**Figure 3** and **Table 7**). These data are significant in that this is the first reported observation for any members of this class of enzyme to be active on herbicides outside the phenoxy auxins. The implications are that this enzyme is promiscuous enough to utilize chemicals with similar phenoxyalkanoate substructures even though they have completely different modes of action as herbicides.

Example 3 – *In vitro* assay of AAD-1 (v1) activity via colorimetric phenol detection

[00199] 3.1 – AAD-1 (v1) assay.

[00200] AAD-1 (v1) enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides such as haloxyfop and cyhalofop (see **Figure 4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily

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detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

[00201] Enzyme assays were performed in a total volume of 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 15 minutes of incubation at 25° C, the reaction was terminated by addition of 10 μl 100 mM sodium EDTA. Color was developed by addition of 15 μl pH 10 buffer (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH), 1.5 μl 2% 4-aminoantipyrine and 1.5 μl 8% potassium ferricyanide. After 10 to 20 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols. Later assays were made more convenient by consolidating the additions as follows: the reaction was quenched by addition of 30 μl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer and 0.2 % 4-aminoantipyrine, then adding 10 μl 0.8 % potassium ferricyanide.

[00202] 3.2 – Extraction.

[00203] Activity of recombinant AAD-1 (v1) expressed in *Escherichia coli*. *E. coli* cell pellets were resuspended in 0.1 M Tris, pH 7.4 + 1mg/ml lysozyme (5 ml/cells from 250 ml culture; 20 ml/cells from 1 liter) at room temperature. After about 15 minutes with occasional shaking, the suspension was frozen in liquid nitrogen then thawed. DNase was added to 0.02 mg/ml final concentration and $MgCl_2$ to 1 mM. After the extract was no longer viscous, the extract was centrifuged for 15 min. The supernatant was passed over a BioRad 10DG column pre-equilibrated with 20 mM MOPS pH 6.75 and the eluant stored in aliquots at –70° C. Assays were either performed with these unpurified desalted extracts or with purified enzymes.

[00204] A cell pellet from a 250 ml culture of induced *E. coli* cells expressing pDAB3203 containing the gene encoding *AAD-1 (v1)* was extracted and assayed using the previously described protocols. The 2,4-D cleaving activity in the AAD-1 (v1) extract was compared to that from *E. coli* cells expressing a vector without *AAD-1 (v1)* using 1 mM 2,4-D and is

shown in **Figure 5**. The amount of 2,4-dichlorophenol formed is clearly proportional to the amount of extract added to the assay whereas the control extract contains no 2,4-D cleaving activity.

[00205] The activity of this extract was tested on four additional herbicides, (R,S)-dichlorprop, (R,S)-mecoprop, (R,S)-haloxyfop and (R,S)-diclofop in comparison to 2,4-D (all at a final concentration of 0.5 mM) using 4 µl of the *E. coli* extract per assay with a 15 min assay period. **Figure 6A** shows that AAD-1 (v1) cleaved all five herbicides to yield a phenol with the relative activity on the substrates being dichlorprop = mecoprop > diclofop > haloxyfop > 2,4-D. Thus AAD-1 (v1) has activity on graminicidal aryloxyphenoxypropionate herbicides as well as phenoxy auxins.

[00206] The AAD-1 (v1) extract was then tested using racemic (R,S)-haloxyfop, the R enantiomer of haloxyfop and the S-enantiomer of cyhalofop (all at 0.5 mM) as potential substrates to ascertain the likely enantiomeric specificity of AAD-1 (v1). The results are shown in **Figure 6B**. The activity of the enzyme on (R)-haloxyfop was equivalent to that on (R,S)-haloxyfop whereas no activity could be seen on the S-enantiomer of cyhalofop indicating that AAD-1 (v1) has R specificity on AOPPs.

Example 4 – Substrate Specificity of AAD-1 (v1)

[00207] 4.1 – Additional substrates of AAD-1 (v1).

[00208] The substrate specificity of AAD-1 (v1) toward a variety of commercial and experimental herbicides was tested. Purified AAD-1 (v1) was used at either 1 or 10 µg per 160 µl assay and each substrate was tested at 1 mM with an assay time of 15 minutes. **Table 3** shows the A_{510} detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced small amounts of phenol from a variety of phenoxyacetates including 2,4-D. The relative rates on these substrates are better gauged from the assays using the higher amounts (10 µg) of AAD-1 (v1). From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates, X188476 and X398166 (sesone).

[00209] **Table 4** shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly

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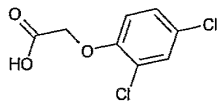
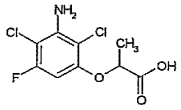
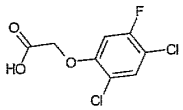
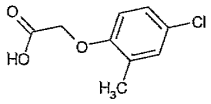
increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

[00210] Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness (**Table 8**). X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast to the close analog 3-amino-dichlorprop, which is ~7- fold better than 2,4-D as a substrate (**Table 3**). The 5-F substitution appears to decrease the effectiveness of X11115427 as a substrate for AAD-1 (v1). The rates of product formation from 5-F-phenoxyacetate and MCPA were 32% and 55% that of 2,4-D respectively.

[00211] **Table 8:** Effect of AAD-1 (v1) on three substrates relative to 2,4-D. Substrates were assayed as in Table 6 at 1 mM using a crude recombinant AAD-1 (v1) extract from *E. coli.*

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Table 8. Effect of AAD-1 (v1) on three substrates relative to 2,4-D.

Registry ID	MOLSTRUCTURE	Compound	A510	% 2,4-D
195517		2,4-D	0.177	100
11115427		(R,S)-3-amino, 5-F-dichlorprop	0.221	125
124987		5-F, 2,4-D	0.056	32
192711		MCPA	0.097	55

[00212] 4.2—Kinetic characterization.

[00213] The K_m and k_{cat} values of purified AAD-1 (v1) (see Example 10) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μM Na ascorbate; 200 μM Fe^{2+}; 1 mM α-ketoglutarate; 25° C). The dose response curves were fitted using Grafit (Erithacus Software, UK), and the graphs and derived constants are shown in **Figure 7** and **Table 9** respectively. The K_m values for the four substrates were fairly similar (75 – 125 μM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in **Table 3** and **Table 4** as these were performed at high (saturating) substrate concentrations (1 mM).

[00214] **Table 9:** Kinetic Constants for AAD-1 (v1) Substrates. Kinetic constants were derived from the data in Figure 7 using Grafit fitting to the Michaelis-Menten equation.

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Table 9. Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (μM) ± SE	V_{max} (μmol min^{-1} mg^{-1} AAD1) ± SE	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
R-dichlorprop	75 ± 10	0.79 ± 0.03	26.1	348
R-quizalofop	125 ± 20	0.57 ± 0.03	18.9	151
R-haloxyfop	120 ± 54	0.34 ± 0.04	11.2	94
2,4-D	96 ± 8	0.57 ± 0.00	2.7	28

[00215] The relative K_m and k_{cat} values for dichlorprop and 2,4-D differ significantly from those published for the R-specific dioxygenase from *Delftia acidovorans* by Westendorf *et al.* (2003) (*Acta Biotechnol.* 23: 3-17). The published k_{cat}/K_m value for 2,4-D is 0.6% that of dichlorprop, whereas in our studies, the k_{cat}/K_m value for 2,4-D is 8% that of dichlorprop. Thus, in this study, AAD-1 (v1) is unexpectedly effective at catalyzing the cleavage of 2,4-D. This increases its potential utility for conferring diverse herbicide tolerance traits in transgenic applications.

[00216] 4.3 – Additional substrates for AAD-1 (v1).

[00217] Three additional substrates were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in **Table 8**. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness. X11115427 was only slightly better (125%) as a substrate than 2,4-D. This is in contrast to 3-aminodichlorprop which is >7 fold better than 2,4-D as a substrate (**Table 3**). Thus, the 5-F substitution has significantly decreased the effectiveness of X11115427 as a substrate for AAD-1 (v1). A similar pattern is seen with 5-F-2,4-D which is only 32% as effective as a substrate relative to 2,4-D. In this assay, MCPA was also less effective as a substrate of AAD-1 (v1) (55% relative to 2,4-D).

Example 5 – Optimization of Sequence for Expression in Plants

[00218] 5.1 – Background.

[00219] To obtain high expression of heterologous genes in plants, it may be preferred to reengineer said genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

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[00220] One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (*e.g.*, TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (*e.g.*, polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

[00221] **Table 10** illustrates how high the G+C content is in maize. For the data in **Table 10**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

Table 10: Compilation of G + C contents of protein coding regions of maize genes

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

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

[00222] Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (*i.e.*, some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A

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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.

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Table 11. Preferred amino acid codons for proteins expressed in maize

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

[00224] It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

[00225] Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled for the gene sequences for the

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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.

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Table 12. Codon composition comparison of native *AAD-1 (v1)* coding region and Plant-Optimized version.

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
ALA (A)	GCA	2	9.1	5	22	LEU (L)	CTA	0	0.0	0	0
	GCC	10	45.5	8	35		CTC	5	22.7	7	32
22	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43	22	CTT	0	0.0	8	36
ARG (R)	AGA	0	0.0	7	30		TTA	0	0.0	0	0
	AGG	0	0.0	7	30		TTG	1	4.5	7	32
	CGA	0	0.0	0	0	LYS (K)	AAA	1	14.3	2	29
23	CGC	16	69.6	5	22	7	AAG	6	85.7	5	71
	CGG	4	17.4	0	0	MET (M)	ATG	8	**100**	8	**100**
	CGT	3	13.0	4	17	PHE (F)	TTC	10	76.9	9	69
ASN (N)	AAC	8	100.0	4	50	13	TTT	3	23.1	4	31
8	AAT	0	0.0	4	50	PRO (P)	CCA	1	5.9	8	47
ASP (D)	GAC	15	78.9	10	53		CCC	9	52.9	1	6
19	GAT	4	21.1	9	47	17	CCG	7	41.2	0	0
CYS (C)	TGC	3	100.0	2	67		CCT	0	0.0	8	47
3	TGT	0	0.0	1	33	SER (S)	AGC	11	73.3	4	27
END	TAA	0	0.0	0	0		AGT	0	0.0	0	0
1	TAG	1	100.0	0	0	15	TCA	0	0.0	4	27
	TGA	0	0.0	1	100		TCC	2	13.3	3	20
GLN (Q)	CAA	0	0.0	7	54		TCG	2	13.3	0	0
13	CAG	13	100.0	6	46		TCT	0	0.0	4	27
GLU (E)	GAA	8	50.0	5	31	THR (T)	ACA	1	4.3	6	26
16	GAG	8	50.0	11	69		ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29	23	ACG	5	21.7	0	0
	GGC	15	71.4	7	33		ACT	1	4.3	8	35
21	GGG	3	14.3	2	10	TRP (W)	TGG	5	**100**	5	**100**
	GGT	3	14.3	6	29	TYR (Y)	TAC	7	70.0	6	60
HIS (H)	CAC	6	60.0	5	50	10	TAT	3	30.0	4	40
10	CAT	4	40.0	5	50	VAL (V)	GTA	2	7.1	0	0
ILE (I)	ATA	0	0.0	3	25		GTC	11	39.3	8	29
12	ATC	12	100.0	5	42	28	GTG	15	53.6	10	36
	ATT	0	0.0	4	33		GTT	0	0.0	10	36
	Totals	148		149			Totals	148		148	

[00228] 5.3 – Completion of binary vectors.

[00229] 5.3.1 – Rebuilt *AAD-1 (v3)*. The plant optimized gene *AAD-1 (v3)* was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:5) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized *AAD-1 (v3)* DNA sequence. The *AAD-1 (v3)* gene was cloned into pDAB726 as an Nco I – Sac I fragment. The resulting construct was designated pDAB720,

containing: [AtUbi10 promoter: Nt OSM 5'UTR: *AAD-1 (v3)*: Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB721, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: *AAD-1 (v3)*: Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: *PAT*: ORF25/26 3'UTR] was restriction digested (with Bam HI, EcoR I, EcoR V, HinD III, Pac I, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

[00230] 5.3.2 – Native *AAD1 (v1)* and modified *AAD-1 (v2)*. The *AAD-1 (v1)* gene (SEQ ID NO:3) was PCR amplified from pDAB3203. During the PCR reaction, alterations were made within the primers to introduce the NcoI and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "rdpA(ncoI)" [CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC] (SEQ ID NO:6) and "3'saci" [GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA] (SEQ ID NO:7) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicenter).

[00231] The PCR amplicon was ligated into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward (SEQ ID NO:16) and M13 Reverse (SEQ ID NO:17) primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents.

[00232] Sequence data identified a clone with the correct sequence. During analysis a superfluous NotI restriction site was identified toward the 3' end of *AAD-1 (v1)*. This site was removed to facilitate cloning into pDAB3038. To remove the additional site a PCR reaction was performed with an internal 5' primer. The NotI site was altered by incorporating a new codon for an amino acid to remove the spurious NotI site. This change would alter the arginine at position 212 to a cysteine. The PCR primers "BstEII/Del NotI" [TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC] (SEQ ID NO:8) and "3' saci" (SEQ ID NO:7) were used.

[00233] A PCR reaction was completed using the Fail Safe PCR System (Epicenter) and the resulting fragment was cloned into the pCR 2.1 TOPO TA cloning kit (Invitrogen). Confirmation of the correct PCR product was completed by DNA sequencing, and the "fixed" gene was given the designation *AAD-1 (v2)* (SEQ ID NO:4).

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[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

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protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in **Table 13.** Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

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

where C1 is the codon in question, C2, C3, *etc.* represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of **Table 13** (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of **Table 13**. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Indiana).

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Table 13. Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

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

***DNU** = Do Not Use

[00238] To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall

weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21.

[00239] The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is given as bases 1-1575 of SEQ ID NO:22. Synthesis of a DNA fragment comprising SEQ ID NO:22 was performed by a commercial supplier (PicoScript, Houston TX).

[00240] 5.3.4 - Cloning of additional binary constructs. The completion of pDAB3295 and pDAB3757 incorporated the use of the GateWay Cloning Technology (Invitrogen, cat #11791-043 and cat #12535-019). The GateWay Technology uses lambda phage-based site-specific recombination to insert a gene cassette into a vector. For more information refer to *Gateway Technology: A universal technology to clone DNA sequence for functional analysis and expression in multiple systems*, © 1999-2003, Invitrogen Corp., 1600 Faraday Ave., Carlsbad, CA 92008 (printed - 2003). All other constructs created for transformation into appropriate plant species were built using similar procedures as above and other standard molecular cloning methods (Maniatis *et al.*, 1982). **Table 14** lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

[00241] The *sacB* gene was added to the binary vector pDAB3289 as a bacterial negative selection marker to reduce the persistence of *Agrobacterium* associated with transformed plant tissue. SacB is a levan-sucrase enzyme produced by *Bacillus* spp. and is toxic to most Gram negative bacteria when grown in the presence of sucrose (Gay *et al.*, 1983). The *sacB* gene was recovered on a *Hind* III fragment from plasmid pRE112 (Edwards, *et al.*, 1998) and cloned into the unique *Hind* III site in pDAB3289.

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Table 14. Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species * Trans-formed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	-	-	-	Erythromycin	-	pat	CsVMV	Agro binary
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	AAD1 v3	CsVMV	Agro binary
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	HptII	AtUbi3	Agro binary
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Aerosol beam
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	-	-	Spectinomycin	-	pat	AtUbi10	Agro binary
3403		Cn, R	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	Same as GOI		Whiskers / Gun
3404		Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	pat	OsAct1	Whiskers
3415	1283	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Ampicillin	-	AHAS v3	OsAct1	Whiskers
3602	1421	Cn	AAD1 v3	ZmUbi1	-	RB7 Mar v2	-	-	Spectinomycin	-	AHAS v3	OsAct1	Agro Superbinary
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	-	pat	AtUbi11	Agro binary
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	-	-	Erythromycin	-	pat	CsVMV	Agro binary

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

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

(721 and 793) Atu ORF1 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 1 3' Untranslated Region
(3295 and 3757) Atu ORF24 3' UTR = *Agrobacterium tumefaciens* Open Reading Frame 24 3' Untranslated Region

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

Example 6 – Transformation into *Arabidopsis* and Selection

[00242] 6.1 – *Arabidopsis thaliana* growth conditions.

[00243] Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, MO) solution. The suspended seed was stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

[00244] Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, WA) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

[00245] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2 – *Agrobacterium* transformation.

[00246] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/L) or spectinomycin (100 mg/L) containing a streaked DH5α colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB + erythromycin). The cultures were incubated overnight at 37° C with constant agitation. Qiagen (Valencia, CA) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

[00247] Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50 μl of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4kV, Pulse length: 5msec.

[00248] After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C in a water bath with constant

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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

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salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044μM benzylamino purine (10 μl/liter of 1 mg/ml stock in DMSO) and 300 μl/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C, with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

[00252] 6.4 – Selection of transformed plants.

[00253] Freshly harvested T_1 seed (transformed with native [*AAD-1 (v2)*] or plant optimized [*AAD-1 (v3)*] gene) was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00254] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00255] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)* (plant optimized gene) or *AAD-1 (v2)* (native microbial gene) to provide phenoxy auxin herbicide resistance.

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[00256] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR_{99} for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200g ae/ha).

[00257] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO). Dichlorprop used was technical grade (Sigma, St. Louis, MO) dissolved in DMSO and diluted in water (<1% DMSO final concentration). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of phenoxy herbicides in 200 mM Tris buffer (pH 9.0) to a final pH of ~7-8.

[00258] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded *in planta*.

[00259] Although *Arabidopsis*, being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, *AAD-1 (v3)*-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above Injury ratings were taken 7 and 14 days after treatment. Likewise, T_1 individuals were treated with the pyridyloxyacetate auxin herbicide, fluroxypyr.

[00260] 6.5 – Results of selection of transformed plants.

[00261] The first *Arabidopsis* transformations were conducted using *AAD-1 (v3)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493

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glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (*see* **Table 15**). A small lot of *AAD-1 (v2)* (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

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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 T_1 individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Codon bias	Total seed sown and screened	Number of resistant T_1	Selection rate	Selection rate range	% of selected plants expressing PAT[3]
Glufosinate[1]	*AAD-1 (v2)*	n	84,000	278	0.33%	0.33%	nd[4]
Glufosinate[1]	*AAD-1 (v3)*	p	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D[2]	*AAD-1(v3)*	p	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate[1]	*AAD-2 (v1)*	n	1,301,500	228	0.018%	0.007 to 0.021%	100%
Glufosinate[1]	*AAD-2(v2)*	p	200,000	224	0.11%	0.11%	nd[4]

[1]Glufosinate selection scheme: 280g ai/ha glufosinate applied 5-6 + 10 DAP
[2]2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP
[3]PAT protein expression determined by PAT ELISA strips
[4]nd, not determined
[5]codon bias, n-native microbial gene, p=plant optimized

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[00262] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** compares the response of native *AAD-1 (v2)* and plant optimized *AAD-1 (v3)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. Both genes imparted resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)* or *AAD-1 (v3)* versus the wildtype or *PAT/Cry1F* transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence *AAD-1 (v3)* versus the native sequence *AAD-1 (v2)* (see **Table 16**). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T_1 individuals expressing AAD-1 (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6x field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

[00263] **Table 16.** *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

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Table 16. *AAD-1 v3* (plant optimized), or *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* response to a range of 2,4-D rates applied postemergence.

	% Injury			% Injury	
Native AAD-1 (v2) gene					Std
Averages	<20%	20-40%	>40%	Ave	Dev
Untreated control-buffer	20	6	7	25.3	34.7
50 g ae/ha 2,4-D	55	16	9	14.8	22.7
200 g ae/ha 2,4-D	45	11	24	34.1	39.3
800 g ae/ha 2,4-D	11	32	37	52.5	34.2
	% Injury			% Injury	
Native AAD-2 gene				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	4	1	1	25.0	21.7
50 g ae/ha 2,4-D	1	2	11	68.2	30.2
200 g ae/ha 2,4-D	0	3	11	82.7	28.8
800 g ae/ha 2,4-D	0	0	14	99.8	0.8
	% Injury			% Injury	
Rebuilt AAD-1 (v3) gene				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	9	0	0	0.0	0.0
50 g ae/ha 2,4-D	10	1	5	24.3	35.9
200 g ae/ha 2,4-D	11	4	1	14.0	25.9
800 g ae/ha 2,4-D	11	4	1	14.7	26.1
	% Injury			% Injury	
Wildtype				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.0	0.0
200 g ae/ha 2,4-D	0	0	15	95.1	0.5
800 g ae/ha 2,4-D	0	0	15	100.0	0.0
	% Injury			% Injury	
PAT/Cry1F (transformed control)				Ave	Std
Averages	<20%	20-40%	>40%		Dev
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.7	4.2
200 g ae/ha 2,4-D	0	0	15	97.2	1.7
800 g ae/ha 2,4-D	0	0	15	100.0	0.0

[00264] **Table 17** shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400 – 1,600 g ae/ha (**Table 18**). Normal field use rate for haloxyfop (a grass-specific

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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 pryridyloxyacetate auxin) in transformed *Arabidopsis*, consistent with *in vitro* work using heterologously expressed enzyme.

[00265] **Table 17.** T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T_1 is an independent transformation event, one can expect significant variation of individual T_1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 17. T_1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence.

AAD-1 v3	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	7	1	0	5.0	7.6	0-20
50 g ae/ha RS-dichlorprop	7	1	0	3.1	8.8	0-25
200 g ae/ha RS-dichlorprop	4	1	3	40.0	50.1	0-100
800 g ae/ha RS-dichlorprop	0	5	3	51.9	40.0	20-100
PAT/Cry1F	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	0	6	2	38.1	25.3	20-95
50 g ae/ha RS-dichlorprop	0	0	8	80.0	25.3	50-100
200 g ae/ha RS-dichlorprop	0	0	8	98.3	2.2	95-100
800 g ae/ha RS-dichlorprop	0	0	8	100.0	0.0	100
Wildtype	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
12.5 g ae/ha RS-dichlorprop	3	0	0	13.3	2.9	10-15
50 g ae/ha RS-dichlorprop	0	0	3	53.3	5.8	50-60
200 g ae/ha RS-dichlorprop	0	0	3	95.0	5.0	90-100
800 g ae/ha RS-dichlorprop	0	0	3	100.0	0.0	100

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[00266] **Table 18.** T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T1 is an independent transformation event, one can expect significant variation of individual T1 responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. *PAT/Cry1F*-transformed *Arabidopsis* served as an auxin-sensitive transformed control. Wildtype *Arabidopsis* is untransformed.

Table 18. T1 *Arabidopsis* response to a range of haloxyfop rates applied postemergence at artificially high rates attempting to show tolerance of the dicot *Arabidopsis* to the graminicide.

AAD-1 v3		% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	3	1	0	6.3	9.5	0-20
800 g ae/ha haloxyfop	1	1	2	46.3	42.7	0-85
1600 g ae/ha haloxyfop	1	0	3	65.0	47.3	0-100
PAT/Cry1F		% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	4	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	4	0	0	10.0	0.0	10
400 g ae/ha haloxyfop	0	4	0	27.5	5.0	20-30
800 g ae/ha haloxyfop	0	0	4	78.8	6.3	70-85
1600 g ae/ha haloxyfop	0	0	4	47.5	43.5	80-100
Wildtype		% Injury			% Injury	
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
Untreated control	3	0	0	0.0	0.0	0
100 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
200 g ae/ha haloxyfop	3	0	0	0.0	0.0	0
400 g ae/ha haloxyfop	0	3	0	20.0	0.0	20
800 g ae/ha haloxyfop	0	0	3	73.3	10.4	70-85
1600 g ae/ha haloxyfop	0	0	3	93.3	11.5	80-100

[00267] 6.6 – *AAD-1 (v3)* as a selectable marker.

[00268] The ability to use *AAD-1 (v3)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed with as described above. T_1 seed

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transformed with *PAT* and *AAD1 (v3)* (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active *PAT* gene.

[00269] Fifty-three out of 70,000 seed planted were identified as resistant to 2,4-D. ELISA was used to screen a subset of 44-individuals from this population for PAT protein expression. Ninety six percent of the individuals were positive indicating the presence of the co-transformed gene, *PAT*. The low number of negative ELISA results (4%) is in line with a 3% error rate in populations of glufosinate-resistant plants (**Table 15**). The efficiency of selection appears to be somewhat less with 2,4-D (0.08%) vs. glufosinate (0.12%); however, the range of selection rates across all experiments would indicate both selection agents are equally good for selecting *Arabidopsis* transformed with *AAD-1 (v3)* or *PAT* genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

[00270] 6.7 – Heritability.

[00271] A variety of T_1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 60% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00272] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show *AAD1 (v3)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

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[00273] 6.8 –Additional herbicide resistance attributable to AAD-1 in *Arabidopsis*.

[00274] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified *in vitro* plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4 homozygous plants id = PAAD1.315.064) were sterilized by agitating for 10 min in a 50% bleach solution. These seeds were then rinsed four times with sterile water to remove the bleach.

[00275] Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration of DMSO never exceeded 1% (v/v). After thorough mixing, a 6 mL aliquot of the warm media containing the appropriate concentration of compound was added to each well of a 6-well, flat bottom, polystyrene tissue culture tray (Falcon 353046, Becton Dickson and Company, Franklin Lakes, NJ). After the media solidified, approximately 20 to 30 *Arabidopsis* seeds were applied on top of the solidified media and the remaining 2 mL of media was poured over the seeds. The plates were lightly agitated to disperse the seeds, covered and allowed to cool until the media had completely solidified. The plates were incubated for 7 days at 25°C under continual fluorescent lighting (75 $\mu E\ m^{-2}\ s^{-1}$). Nutrient Media Composition was as described in Example 2.2 and in Somerville and Orgen (1982).

[00276] Assessment of growth reduction. The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in **Table 19**. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All

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commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported *in planta*. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of *AAD-1*-transformed commercial crops or experimental plant species.

Table 19. *In vitro* plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

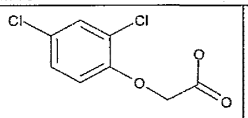
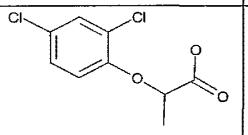
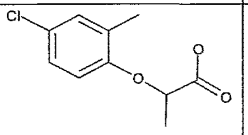
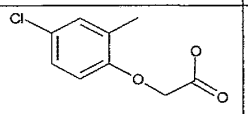
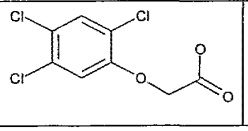
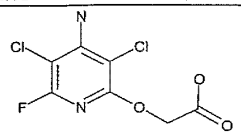
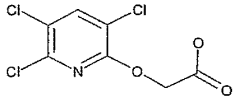
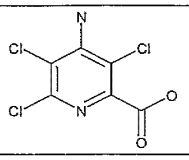
Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Phenoxy auxins							
2,4-D		0.2	10	< 0.01	0.04	50	> 4
dichlorprop		2	5	0.01	1.5	2.5	150
Mecoprop		2	25	0.01	1.5	12.5	150
MCPA		0.2	1	0.01	0.03	5	3
2,4,5-T		1.5	10	<0.01	<0.01	6.67	NA

Table 19. *In vitro* plate test assessment of herbicide substrate cross resistance afforded by AAD-1 (v3) in homozygous T_4 *Arabidopsis* (ARBTH).

Compound	Structure	wt ARBTH shoot I50	PAAD1.315.064 T3 ARBTH shoot I50	wt ARBTH root I50	PAAD1.315.064 ARBTH root I50	shoot ratio	root ratio
		ppm					
Pyridine auxins							
Fluroxypyr		2	1	0.2	0.2	0.5	1
Triclopyr		0.2	0.04	0.02	0.02	0.2	1
Picloram		1	0.5	0.3	0.15	0.5	0.5

[00277] 6.9 – Foliar applications herbicide resistance in AAD-1 *Arabidopsis* .

[00278] The ability of *AAD-1 (v3)* to provide resistance to other aryloxyphenoxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates described in Example 6.8. T_4 generation *Arabidopsis* seed, homozygous for *AAD-1 (v3)* (line AAD1.01.315.076) was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed from a range of phenoxy auxin herbicides: 12.5-1600 g ae/ha 2,4-D dimethylamine salt (DMA) (Riverside Chemicals), 12.5-1600 g ae/ha mecoprop (AH Marks), 50-3200 g ae/ha R-dichlorprop (AH Marks), 8.75-1120 g ae/ha 2,4,5-T (technical grade); pyridyloxyacetates herbicides 50-3200 g ae/ha triclopyr (Dow AgroSciences) and 50-3200 g ae/ha fluroxypyr (Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-1 activity, 2,4-dichlorophenol (DCP, Sigma) (at 50-3200 g ae/ha, technical grade). All applications were formulated in 200 mM Hepes buffer (pH 7.5). Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment and are averaged over two experiments.

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[00279] These results (see **Table 20**) confirm that *AAD-1 (v3)* in *Arabidopsis* provides robust resistance to the phenoxyacetic auxins, phenoxypropionic auxins, but have not shown significant cross resistance to the pyridyloxyacetic auxins tested and corroborates the *in vitro* enzyme and whole plate substrate specificity data. Additionally, there is no effect of the metabolite, 2,4-dichlorphohenol (DCP), on wildtype or transgenic *Arabidopsis*.

Table 20. Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Phenoxypropionic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**PatCry1f - Control**
50 g ae/ha R-Dichlorprop	3	31
200 g ae/ha R-Dichlorprop	3	73
800 g ae/ha R-Dichlorprop	3	89
3200 g ae/ha R-Dichlorprop	3	95
12.5 g ae/ha Mecoprop	3	0
25 g ae/ha Mecoprop	0	2
50 g ae/ha Mecoprop	0	17
100 g ae/ha Mecoprop	0	33
200 g ae/ha Mecoprop	3	62
400 g ae/ha Mecoprop	0	78
800 g ae/ha Mecoprop	0	93
1600 g ae/ha Mecoprop	0	100
Phenoxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Cry1f - Control**
12.5 g ae/ha 2,4-D DMA	0	67
25 g ae/ha 2,4-D DMA	0	78
50 g ae/ha 2,4-D DMA	0	93
100 g ae/ha 2,4-D DMA	0	100
200 g ae/ha 2,4-D DMA	0	100
400 g ae/ha 2,4-D DMA	0	100
800 g ae/ha 2,4-D DMA	0	100
1600 g ae/ha 2,4-D DMA	0	100
8.75 g ae/ha 2,4,5-T	0	0
17.5 g ae/ha 2,4,5-T	3	20
35 g ae/ha 2,4,5-T	0	43
70 g ae/ha 2,4,5-T	3	85
140 g ae/ha 2,4,5-T	0	95
280 g ae/ha 2,4,5-T	0	98
560 g ae/ha 2,4,5-T	17	100
1120 g ae/ha 2,4,5-T	3	100

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Table 20. Comparison of homozygous T_4 AAD-1 (v3) and wildtype *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Pyridyloxyacetic auxins		
	Ave % Injury 14DAT	
Herbicide Treatment	**AAD1.01.315.076.T_4 homozygous AAD1 plants**	**Pat/Cry1f - Control**
50 g ae/ha Triclopyr	31	36
200 g ae/ha Triclopyr	58	65
800 g ae/ha Triclopyr	74	84
3200 g ae/ha Triclopyr	97	95
50 g ae/ha Fluroxypyr	48	76
200 g ae/ha Fluroxypyr	75	85
800 g ae/ha Fluroxypyr	88	85
3200 g ae/ha Fluroxypyr	95	95
	Inactive DCP metabolite	
50 g ae/ha 2,4-DCP	0	0
200 g ae/ha 2,4-DCP	0	0
800 g ae/ha 2,4-DCP	0	0
3200 g ae/ha 2,4-DCP	0	0

[00280] 6.10 – Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

[00281] An experiment was designed to examine if the level of AAD-1 (v3) expression in *Arabidopsis* varies at different growth stages. A high-tolerance, homozygous, *AAD-1 (v3)* T_4 line (id=PAAD1.01.345.163) was grown in greenhouse. Half of the plants were treated with 800 g ae /ha of 2,4-D (as previously described) while the other half were not treated. Two leaves, the 3rd leaf from the top and the 5th leaf from the bottom, were harvested from 5 plants, both treated and untreated, and analyzed by ELISA and Western Blotting (as described in Example 11) experiments at 4, 10, 14, 20 and 25 DAT. **Figures 8A** and **8B** showed that there was statistically no difference in AAD-1 (v3) expression between young and old leaves. In addition, the herbicide 2,4-D had little impact on the expression level of AAD-1 (v3) protein. The protein levels accumulated in older plants with some significant protein degradation at the later time points.

[00282] In a separate experiment, four different homozygous T_4 lines of *Arabidopsis* displaying different tolerance level to the herbicide 2,4-D were sprayed with various levels (0, 200, 800 and 3200 g/ha) of 2,4-D and their herbicide injury and AAD-1 (v3) expression were examined. Four days after the herbicide treatment, little injury was observed in three of the four lines, even at the highest dose tested (**Figure 9A**). These plants also expressed high

level of AAD-1 (v3), from 0.1 to 0.25% of (**Figure 9B**). On the contrary, the low tolerance line expressed less than 0.1% of AAD-1 (v3) in TSP and suffered observable injury. More importantly, they recovered from the injury on 14 DAT (**Figure 9A**), indicating that the low level of AAD-1 (v3) expression was able to protect the plants from the serious herbicide damage. All control plants suffered serious injury and died 14 DAT at doses 800 g ae/ha 2,4-D and above.

[00283] 6.11 – Molecular analysis of *AAD-1 (v3) Arabidopsis*.

[00284] Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number and/or Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple *AAD-1 (v3)* homozygous lines to determine stable integration of the plant transformation unit containing *PAT* and *AAD-1 (v3)*. Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

[00285] For Southern analysis, a total of 1 μg of DNA was subjected to an overnight digest of Nsi I for pDAB721 to obtain integration data. The samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20SSC was then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of *PAT*. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then

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subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at -80° C.

[00286] Results showed all 2,4-D resistant plants assayed contained *PAT* (and thus by inference, *AAD-1 (v3)*). Copy number analysis showed total inserts ranged from 1 to >10 copies. This correlates, too, with the AAD-1 (V3) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance (>>200 fold) to all commercially available phenoxyacetic and phenoxypropionic acids.

[00287] 6.12 – *Arabidopsis* transformed with molecular stack of *AAD-1 (v3)* and glyphosate resistance gene.

[00288] T_1 *Arabidopsis* seed was produced, as previously described, containing pDAB3230 plasmid (*AAD-1 (v3)* + *EPSPS*) coding for a putative glyphosate resistance trait. T_1 transformants were selected using *AAD-1 (v3)* as the selectable marker as described in example 6.6, except the 2,4-D rate used was 75 g ae/ha. Twenty-four T_1 individually transformed events were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, *AAD-1 (v3)* + *PAT* T_5 homozygous lines (pDAB721-transformed), and *PAT* + *Cry1F* homozygous line (transformed control). Only pDAB3230 plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in 200 mM Hepes buffer (pH 7.5). All treatments were replicated 4 or 5 times. Plants were evaluated 7 and 14 days after treatment. I_{50} values were calculated and show >14 fold level of tolerance imparted by *EPSPS* molecularly stacked with *AAD-1 (v3)* (*see* **Figure 10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate + 2,4-D treatments applied in tankmix as described in Example 21 and shown for *AAD-1 (v3)*-transformed corn in Example 8.

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Example 7 – WHISKERS-Mediated Transformation Into Maize, and Use of *AAD-1 (v3)* as a Selectable Marker

[00289] 7.1 – Cloning of *AAD-1 (v3)*.

[00290] The *AAD-1 (v3)* fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and SacI and the 5175 bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 (OsAct1/PAT/ZmLip) were digested with NotI. The 3442 bp band from pDAB3403 and the 11017 bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5α, and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404, which contains ZmUbi1/po-aad1/ZmPer5::OsAct1/PAT/ZmLip.

[00291] 7.2 – Callus/suspension initiation.

[00292] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00293] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu *et al.*, 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

[00294] To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS

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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

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liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00303] After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker for 2 hours before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

[00304] Either 3 or 6 mL of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60 x 20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C in a dark box that was loosely sealed with a single layer of plastic (<2 mils thick) to minimize evaporation of the individual plates.

[00305] After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (1H) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 1.0 mg/L bialaphos; 2.5 g/L Gelrite, pH 5.8) or GN6D (1H) medium (same as GN6 (1H) except with 8.0 mg/L dicamba and 0.8 mg/L 2,4-D).

[00306] Plates were placed in boxes and cultured as before for an additional week. Two weeks post-transformation, the tissue was embedded by scraping either ½ the cells on the plate or else all cells on the plate into 3.0 mL of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L *myo*-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C) containing 1 mg/L bialaphos. The tissue was broken up and the 3 mL of agarose and tissue were evenly poured onto the surface of a 100 x 15 mm plate of GN6 (1H) or GN6D (1H) medium. This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured for 1 week at 28° C in dark boxes.

[00307] Putatively transformed isolates were typically first visible 5-8 weeks post-transformation. Any potential isolates were removed from the embedded plate and transferred to fresh selection medium of the same concentration in 60 x 20 mm plates. If sustained

growth was evident after approximately 2 weeks, an event was deemed to be resistant and was submitted for molecular analysis.

[00308] Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (1H), containing 1 mg/L bialaphos, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week then higher light (40 $\mu Em^{-2}s^{-1}$) for another week before being transferred to regeneration medium, 36 (1H), which was identical to 28 (1H) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150 x 25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L *myo*-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

[00309] 7.6 – WHISKERS-mediated transformation using haloxyfop selection.

[00310] DNA delivery parameters for direct selection on "fops" were identical to the bialaphos selection procedure except that 85 µg of pDAB3403 and 85 µg of construct containing a GFP (Green Fluorescent Protein) reporter gene were co-transformed together, and only 3 mL of suspension was filtered onto GN6 medium following the 2-hour recovery.

[00311] After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60x20-mm plates of GN6 medium containing 2 mg/L 2,4-D plus 50, 100, or 200 nM R-haloxyfop acid. Plates were placed in boxes and cultured for one additional week. After one week, the tissue was embedded by scraping all cells from the plate into 3.0 mL of melted GN6 agarose medium containing the same concentration of selection agent as in the previous transfer. All steps afterward were identical to the PAT selection/regeneration protocol except that 100 nM R-haloxyfop acid was included in the regeneration media instead of 1 mg/L bialaphos.

[00312] 7.7 – Results. Multiple experiments testing various levels of haloxyfop and cyhalofop were initiated, and 47 isolates were recovered from direct selection. A subset of the callus events were submitted for screening using PCR and Western analyses. Following these expression data, 21 lead events were submitted for Southern analysis. Results using NcoI, a unique cutter, to obtain integration data following probing with *AAD-1 (v3)*, unequivocally demonstrate stable integration of *AAD-1 (v3)* following Whiskers-mediated transformation coupled with "fop" selection.

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[00313] 7.8 – Quantitative demonstration of *in vitro* tolerance from AAD-1 (v3) expressing callus events from bialaphos selection

[00314] Ninety-seven callus isolates recovered from bialaphos selection were submitted for PAT copy number via Invader analysis and *AAD-1 (v3)* PTU analysis via PCR (see Example 7.10). AAD-1 (v3) protein expression using Western blot/Sandwich ELISA (Example 11) was completed on a subset of the events. A summary is described in **Table 21** below. At least 15 T_0 plants were regenerated from each of these events and sent for spray testing and seed production.

Table 21.

Event	PAT Copy #	PCR for *AAD-1 (v3)* PTU	Callus Western
3404-001	2	+	+
3404-006	2	+	+
3404-013	3	+	+
3404-017	1	+	+
3404-020	3	+	nd
3404-022	2	+	+
3404-025	2	+	+
3404-027	3	+	+
3404-031	1	+	+
3404-033	2	+	nd
3404-036	3	+	+
3404-044	3	+	+
3404-050	3	+	+
3404-053	3	+	+
3404-074	2	+	+
3404-082	2	+	+

[00315] A smaller subset of these events was assessed in dose-response studies in comparison to a non-transformed control. A range of concentrations of haloxyfop (from Gallant Super formulation), up to 400 nM, were tested. Dose response data for one event, 3404-006, was generated using the general methods of Example 7.4, and is shown in **Figure 13**. This demonstrates that event 3404-006 showed no significant reduction in callus growth at haloxyfop concentrations up to 400 nM whereas non-transgenic corn callus tissue growth was inhibited at this rate. These data have not been normalized to account for inherent growth differences that are not related to the expression of the transgene.

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[00316] 7.9 –WHISKERS-mediated transformation using imazethapyr selection.

[00317] The ZmUbi1/*AAD-1 (v3)*/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the *AAD-1 (v3)* and *AHAS* Whiskers transformation vector pDAB3415, which is also referred to as pDAS1283) Once completed, this construct was transformed into maize via silicon carbide whiskers-mediated transformation as described in Example 7.4 except 2 mL of cells were filtered, followed by a 7-day recovery on GN6 medium followed by selection on media containing 3 μM imazethapyr from Pursuit® DG herbicide. Following Invader analysis, 36 events were identified that contained the AAD-1 (v3) and the AHAS genes.

[00318] Fifty-three corn calli events from these transformations were tested in both ELISA and Western Blotting experiments for their AAD-1 (v3) expression – a subset of the data is shown below. For the events listed in **Table 22,** the expression levels of those detected positive ranged from 90 to over 1000 ppm of total soluble proteins.

Table 22.		
AAD-1 Event ID Number	**Expression Level (ppm)**	**Western Blot**
1283[1]-001	2	-
1283[1]-002	206	+++
1283[1]-003	1	-
1283[1]-004	90	+++
1283[1]-005	1	-
1283[1]-006	105	+++
1283[1]-007	212	++
1283[1]-008	114	±
1283[1]-009	305	+
1283[1]-010	2	-
1283[1]-011	4	-
1283[1]-012	200	+++
1283[1]-013	134	+++
1283[1]-014	4	-
1283[1]-015	194	+++
1283[1]-016	4	-
1283[1]-017	196	+++
1283[1]-018	3	-
1283[1]-019	178	+
1283[1]-020	260	++
1283[1]-021	144	+++
1283[1]-022	140	+++
1283[1]-023	191	+++
1283[1]-024	392	++
1283[1]-025	368	++
1283[1]-026	14	-
1283[1]-027	1006	++
Neg Control	3	-
Neg Control	3	-
Standard (0.5 µg/mL)		++
Standard (5 µg/mL)		++++

[00319] 7.10– Molecular Analysis: Maize Materials and Methods.

[00320] 7.10.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00321] 7.10.2 – Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and $MgCl_2$ (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63° C for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis was used to identify the estimated copy of the unknown events.

[00322] 7.10.3 – Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the *AAD-1 (v3)* PTU were (Forward – ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:25) and (Reverse – CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:26). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 63° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. Primers for Coding Region PCR *AAD-1 (v3)* were (Forward – ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse – CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 65° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

[00323] 7.10.4 – Southern blot analysis. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µg of DNA was subjected to an overnight digestion of Afl II and also EcoRV for pDAB3404, NcoI for pDAB3403, and SpeI for pDAB1421 to obtain integration data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85 % agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20x SSC was then set up to

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obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using the above coding region PCR using plasmid pDAB3404. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at -80° C.

Example 8 – *In vivo* tolerance and field tolerance data generated from *PAT*-selected (pDAB3404) *AAD-1 (v3)* events

[00324] 8.1 - Tolerance of T_0 corn plants to AOPP herbicides.

[00325] If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (*i.e.*, plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light: 8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure® II (DuPont), Clincher* (Dow AgroSciences), or Gallant Super* (Dow AgroSciences) for quizalofop, cyhalofop, or haloxyfop, respectively. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height, and all sprays contained 1% v/v Agridex crop oil concentrate adjuvant. The number of clones of each event varied from week to week due to the rate of regeneration and acclimation of each event. Overall, an attempt was made to treat representative clones of each event with a range of herbicide doses ranging from 1X

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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.

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Table 23. Selected AAD-1 (v3)-transformed T0 corn events response to three AOPP herbicides applied postemergence.

Construct	Event	Clone	Haloxyfop* g ae/ha	Cyhalofop** g ae/ha	Quizalofop*** g ae/ha	% Injury 14 DAT
3404	001	016	8.8			0
3404	001	018		62.5		0
3404	001	017			8.8	0
3404	001	019			8.8	30
3404	001	020			35	0
3404	017	018			35	0
3404	017	019			35	0
3404	017	020			70	0
3404	017	021			70	0
3404	017	022			140	0
3404	017	023			140	30
3404	017	024			280	30
3404	017	025			280	20
3404	022	019	8.8			0
3404	022	020	17.5			0
3404	022	016	17.5			0
3404	022	024		62.5		0
3404	022	018		125		0
3404	022	021			8.8	0
3404	022	017			17.5	0
3404	022	022			35	0
3404	022	023			70	0
3404	033	012			35	0
3404	033	013			70	0
3404	033	014			70	0
3404	033	015			140	0
3404	033	016			280	0
3404	038	016	8.8			0
3404	038	018		62.5		0
3404	038	017			8.8	0
3404	038	019			35	70
3404	038	020			35	80
3404	038	021			70	80
3404	038	022			70	80
			(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 g ae/ha)	(lethal dose = 4.4 g ae/ha)	

*Gallant super[#] + 1% COC (v/v)
**Clincher [#] + 1% COC (v/v)
***Assure II + 1% COC (v/v)
[#]Trademark of Dow AgroSciences, LLC

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8.2 – Field tolerance of pDAB3404 T_1 corn plants to quizalofop 2,4-D, and glufosinate herbicides.

[00330] Two field trials were established at field stations in Hawaii and Indiana. Corn seed from inbred T_1 plants were utilized to evaluate sixteen AAD1 event lines for tolerance against quizalofop and 2,4-D. Three non-transformed hybrids were included for comparison purposes. The hybrid Hi-II x 5XH571 is of the same parentage as the *AAD-1 (v3)* event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

[00331] The experimental design was a split-plot with four replications. The main plot was herbicide treatment and the sub-plot was *AAD-1 (v3)* event or comparison hybrid. Plots were one row by 3.7 meters with approximately twenty-five seeds planted in each row. For AAD1 events, seeds from a different lineage within the event were planted in each replicate.

[00332] Glufosinate at 560 g ai/ha was applied to *AAD-1 (v3)* plots at the V2 stage to eliminate non-transformed plants. Experimental treatments included commercial formulations of quizalofop applied at 70 and 140 g ae/ha, 2,4-D (dimethylamine salt) at 560 and 1120 g ae/ha, and an untreated control. Treatments were applied using backpack broadcast boom equipment delivering 187 L/ha carrier volume at 130-200 kpa pressure. Quizalofop treatments were applied at the V3-V4 corn stage and 2,4-D treatments were applied at the V5-V6 stage.

[00333] Quizalofop treated plots were visually assessed for crop injury at one and three weeks after application (WAA) using a 0-100% scale, where 0 equals no injury and 100 equals complete death. 2,4-D treated plots were visually assessed for plant leaning at 2 days after application (DAA) using 0-100% scale where 0 equals no leaning from any plant and 100 equals all plants prone. Additionally, 2,4-D plots were visually assessed at 3-4 WAA for brace root deformation using a 0-10 scale.

[00334] 8.2.1 – Results.

[00335] *AAD-1 (v3)* event response to the highest rates tested of quizalofop and 2,4-D are shown in **Table 24**. These rates represent approximately twice the normal commercial use rates. Non-transformed hybrids were severely injured (80-100%) by quizalofop at 70 g ae/ha including the sethoxydim resistant line, although it displayed slightly better tolerance than the other two hybrids. All *AAD-1 (v3)* events except one lineage of event 3404.001 displayed excellent tolerance to quizalofop at 70 g ae/ha. No visible symptoms were observed on the *AAD-1 (v3)* events except with the events noted above.

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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).

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[00339] These results indicate that most *AAD-1 (v3)* transformed event lines displayed a high level of resistance to quizalofop and 2,4-D at rates that were lethal or caused severe epinastic malformations to non-transformed corn hybrids. *See also* **Figure 16.**

[00340] 8.2.2- Expected Mendelian segregation ratios on three T_2 events. Plants from individual lineages of each event were randomly self-pollinated in the field. T_2 seed were hand harvested at physiological maturity. Based on single gene copy number (see **Table 24** above) and overall performance in T_1 generation (segregation, herbicide tolerance, and vigor), three events (022, 031, and 074) were chosen for further evaluation in the field. Breeding rows of each event were planted using a precision cone planter each consisting of 2500-3000 seeds. At the V2 growth stage, all *AAD-1 (v3)* lines were sprayed with 140 g ae/ha quizalofop (Assure® II) using a backpack sprayer as previously described. This rate rapidly killed all "null" (untransformed) segregants. Each event had a segregation ratio consistent with Mendelian inheritance of a single locus, dominant gene (3 resistant:1 sensitive, or 75% survival) (see **Table 24**). Homozygotes and hemizygotes from event 74 were identified by zygosity testing (refer to *AAD-1 (v3)* Invader assay description for corn). Hemizygous plants were removed and homozygous *AAD-1 (v3)* plants were crossed with BE1146 corn inbred introgressed and homozygous for glyphosate resistance trait, NK603, creating a homogeneous F_1 hybrid seed that is hemizygous for glyphosate resistance, *AAD-1 (v3)*, and glufosinate resistance.

[00341] 8.3 – Stacking of *AAD-1 (v3)* and *PAT* with glyphosate resistance genes in corn.

[00342] Homozygous T_2 *AAD-1 (v3)/PAT* corn plants were crossed with glyphosate resistant corn plants producing F_1 seed containing *AAD-1 (v3)*, *PAT*, and glyphosate resistance genes as described in the previous example.

[00343] F_1 seeds were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium (Sun Gro Horticulture). The pots were initially subirrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were subirrigated with deionized water.

[00344] Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. The plants were sprayed with rates of 2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200

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mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

[00345] At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Results of the study at 14 DAT can be seen in **Table 25.**

Table 25.

	Field Rate	% Injury at 14 DAT	
		Hi II X 5XH751	RR/PAT/AAD1
		Ave	Ave
Untreated control	-	0	0
840 g ae/ha glyphosate	1X	98	0
1680 g ae/ha glyphosate	2X	100	0
3360 g ae/ha glyphosate	4X	100	0
560 g ae/ha 2,4-D DMA	1X	10	0
1120 g ae/ha 2,4-D DMA	2X	14	0
2240 g ae/ha 2,4-D DMA	4X	29	0
470 g ae/ha glufosinate	1X	80	0
940 g ae/ha glufosinate	2X	90	3
1880 g ae/ha glufosinate	4X	96	15
840 g ae/ha glyphosate + 560 g ae/ha 2,4-D DMA	1X + 1X	96	1
1680 g ae/ha glyphosate +1120 g ae/ha 2,4-D DMA	2X + 2X	100	2
3360 g ae/ha glyphosate + 2240 g ae/ha 2,4-D DMA	4X + 4X	100	1
470 g ae/ha glufosinate + 560 g ae/ha 2,4-D DMA	1X + 1X	89	5
940 g ae/ha glufosinate + 1120 g ae/ha 2,4-D DMA	2X + 2X	91	10
1880 g ae/ha glufosinate + 2240 g ae/ha 2,4-D DMA	4X + 4X	97	13
840 g ae/ha glyphosate + 470 g ae/ha glufosinate	1X + 1X	90	5
1680 g ae/ha glyphosate + 940 g ae/ha glufosinate	2X + 2X	98	15
3360 g ae/ha glyphosate + 1880 g ae/ha glufosinate	4X + 4X	100	15

[00346] This study demonstrated that the *AAD-1 (v3)* gene in corn can be stacked with a glyphosate resistance gene and a glufosinate resistance gene to provide robust field-level tolerance to 2,4-D, glyphosate, and glufosinate alone or in tank mix combinations.

[00347] 8.3.1 – Resistance of *AAD-1 (v3)* corn using a tank mix of 2,4-D DMA and quizalofop. T_2BC_1 seeds of hemizygous event number 3404-025.001R/R001 Bulked.001.S058 were planted individually into 3-inch pots prepared with Metro-Mix® 360 growing medium. The pots were initially sub-irrigated with Hoagland's solution until wet, then allowed to gravity drain, and grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. For the remainder of the study the plants were sub-irrigated with de-ionized water.

[00348] Plants were allowed to grow until V1 stage in the greenhouse. At this point the plants were selected with 560 g ae/ha Assure® II with the addition of 1% Agridex crop oil concentrate in 200 mM Hepes buffer with the research track sprayer set at 187 L/ha. Plants were allowed 4 days to show symptoms of the selection. All plants were uninjured. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 18-in spray height. All applications were formulated in 200 mM Hepes buffer (pH 7.5) with the addition of 1% v/v Agridex.

At 3 and 14 days after treatment (DAT) plants were evaluated. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Plants from this particular lineage had 0% injury at 14 DAT for all tank mixed combinations, while the wild-type had 100% injury. These results indicate AAD-1 (v3) not only provides robust field level resistance to 2,4-D and quizalofop individually, but also to exaggerated rates of multiple combinations of the two chemistries. One could logically expect to implement novel weed control measures with combinations of phenoxy auxins and AOPP graminicides in corn (or other crops transformed with AAD-1) not previously enabled by a single gene HTC.

[00349] 8.3.2 - Tolerance of (pDAB3403) T_0 corn plants to quizalofop herbicide. A target of approximately eight T_0 plant clones from each of 17 events were regenerated and transferred to the greenhouse for preliminary tolerance screening with postemergence-applied discriminating rate of quizalofop herbicide applied by track sprayer at 35 g ae/ha (1X field rate, 4X lethal dose) to 3-leaf, greenhouse-adapted T_0 corn plants using track sprayer conditions previously described. Plants were rated as Resistant or Sensitive 7 days after treatment. Control, non-transgenic corn was included with each spray application. Two events, Event 014 and 047, had two or more T_0 clones sensitive to 35 g ae/ha quizalofop, indicating an unexpected level of sensitivity for this event. The 15 other events showed stable integration, protein expression, and the ability to tolerate a 4X lethal dose of quizalofop at the whole plant level.

[00350] 8.3.3 Expression of AAD-1 (v3) with respect to quizalofop tolerance.

[00351] Three different T2 lineages from 3404 transformations that were pre-screened with Liberty® (as described previously) to remove nulls were chosen to compare their tolerance to quizalofop with respect to their AAD-1 (v3) expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT (see **Figure 15.**). The highest tolerance line, event

3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1X and higher field rates. This data concludes that corn expressing AAD-1 (v3) can be protected from quizalofop injury at the highest level tested (2,240 g/ha), which is 16 times the 1X field dose of 35 g/ha. In addition, the expression level was consistent throughout the period of the experiment.

Example 9 - *Agrobacterium*-Mediated Transformation of Maize with *AAD-1 (v3)*

[00352] 9.1 - Plant material.

[00353] Seeds of a "High II" (*i.e.*, Parent A and B) F_1 cross (Armstrong *et al.*, 1991) are planted directly into 5 gallon-pots containing 95:5 Metro-Mix® 360: Mineral soil. The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps.

[00354] 9.2 - Tissue source.

[00355] For obtaining immature Hi-II (F2) embryos, controlled sib-pollinations were performed. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. Immature embryos were isolated as described in Example 7.2.

[00356] 9.3 - Preparation of a superbinary vector.

[00357] Construction of an *Agrobacterium* construct, pDAB2272, containing the *AAD-1 (v3)* gene in combination with the AHAS selectable marker gene was accomplished by isolating the 3443 base pair NotI fragment from pDAB3404 containing ZmUbi1 v2/ *AAD-1 (v3)*/ZmPer5 v2 and inserting it into the NotI site of pDAB8549. The resulting plasmid contains the ZmUbi1 v2/ *AAD-1 (v3)*/ ZmPer5 v2 and the OsAct1 v2/AHAS v3/ZmLip v1 cassettes flanked by non-identical MAR regions in the direct orientation. This was subsequently transformed into LBA4404/pSB1 to create the superbinary vector, which was named pDAB3602 but was also referred to as pDAS1421.

[00358] 9.4 - Bacterial supply.

[00359] All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Patent No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The

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bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~ $1x10^9$ cfu/ml) and 100 µM actetosyringone added to the solution.

[00360] 9.5 - Infection and cocultivation.

[00361] The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod liquid medium. Each tube, containing ~100 embryos, is vortexed for 3-5 sec. The medium is removed and replaced with fresh liquid medium and the vortex is repeated. The liquid medium is again removed and this time replaced with an *Agrobacterium* solution at the Klett 200 concentration. The *Agrobacterium* and embryo mixture is vortexed for 30 sec. Following a 5 minute incubation at room temperature, the embryos were transferred to LS-As Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L $AgNO_3$,1, 100 µM actetosyringone, 3.0 g/L Gelrite, pH 5.8) for a 5-day co-cultivation at 25° C.

[00362] 9.6 - Dose response using immature embryos.

[00363] Dose response studies were initiated using immature embryos treated with *Agrobacterium* strain LBA4404 lacking a plasmid as described previously. Once treated, embryos were allowed to co-cultivate for 5 days at 25° C and were then transferred to selection media containing various levels of R-haloxyfop or R-cyhalofop. Embryos were also transferred to media containing 1 mg/L bialaphos and 100 nM imazethapyr as negative controls. Embryos were scored for % embryogenic callus formation after 2 weeks, and then again after 4 weeks. Embryos were tested on R-haloxyfop levels up to 30 nM; however, insufficient reduction of callus formation was seen at the highest levels, so higher concentrations (50 – 100 nM) were used during transformation experiments. Data from embryos grown on cyhalofop-containing media is shown in **Figure 17**.

9.7 – Selection.

[00364] After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then

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moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the *AHAS* gene is used, based on U.S. Patent No. 5,731,180. Tissue was transferred at biweekly intervals on the same medium until embryogenic colonies were obtained. These colonies were maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies were bulked up by transferring to fresh selection medium at 2-week intervals for regeneration and further analysis.

[00365] 9.8. – Regeneration and seed production.

[00366] For regeneration, the cultures are transferred to 28 "induction" medium and 36 "regeneration" medium as described previously containing either 100 nM R-haloxyfop or 1.5 μM cyhalofop for differentiation of plantlets. When plantlets were established, they were transferred to SHGA tubes to allow for further growth and development of the shoot and roots as described previously. Controlled pollinations for seed production were conducted as described previously.

[00367] 9.9 - Event recovery and analysis particulars; whole plant screening of T_0 corn lineages containing *AAD-1 (v3)* and *AHAS* (pDAS1421).

[00368] Seventy-two *Agrobacterium*-transformed events were selected on various levels of R-haloxyfop acid and R-cyhalofop acid *in vitro*. Twenty-two callus samples were analyzed by Southern blot analysis for stable integration of *AAD-1 (v3)* into the genome as described previously. Ten single copy events, as indicated in **Table 26,** were chosen to be regenerated.

Table 26.

Corn event	*In vitro* Selection agent	Southern Copy # (callus)	Western Blot (AAD-1) T_0 Callus	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop	
	(nM)	AAD-1		Resistant	Sensitive
1421[21]-016	50 Haloxyfop	1	+	8	0
1421[22]-020	100 Haloxyfop	1	++	8	0
1421[22]-022	100 Haloxyfop	1	+	8	0
1421[22]-023	100 Haloxyfop	1	++	8	0
1421[3]-036	100 Haloxyfop	1	++	8	0
1421[4]-031	300 Cyhalofop	1	++	9	0
1421[4]-032	300 Cyhalofop	1	++	8	0
1421[4]-033	300 Cyhalofop	1	++	12	0
1421[4]-034	300 Cyhalofop	1	++	8	0
1421[4]-035	300 Cyhalofop	1	++	6	0
Events with more than 1 copy were not taken to the greenhouse					

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[00370] A minimum six regenerated clonal lineages per event were moved to soil in the greenhouse and screened using a track sprayer as previously described to apply 35 g ae/ha quizalofop when 2-4 new, normal leaves had emerged (see section 8.3.3). Presence of AAD-1 protein on a Western blot correlated perfectly with herbicide resistance in the T_0 generation regardless of which AOPP herbicide was used for selection. There is no negative impact of the second HTC gene (*AHAS*) on the function of the *AAD-1 (v3)*.

Example 10 – Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

[00371] All operations during purification were carried out at 4° C. Frozen or fresh *E. coli* cells from approximately 1 L culture, grown and induced as in Example 3, were re-suspended in 200 ml of extraction buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 2 ml of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The soluble extract was obtained by centrifugation in a GSA rotor (Sorvall) at 12,000 rpm (24,000g) for 20 minutes. The supernatant was then loaded onto a Mono Q ion exchange column (Pharmacia HR 10/10) equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 8.0, and the column was washed with same buffer for 10 CV (80 ml). The protein was eluted with 80 ml of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 ml fractions were collected. The fractions containing AAD-1 (v1) were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl, 0.15 M NaCl, and 1 mM DTT, pH 8.0 at a flow rate of 1 ml/min. Purification procedures were analyzed by SDS-PAGE, and protein concentration was determined by Bradford assay using bovine serum albumin as standard.

Example 11 – Recombinant AAD1 Purification and Antibody Production

[00372] Plasmid pDAB3203 containing the *AAD-1 (v1)* gene was maintained frozen at -80° C in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 µL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were

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scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000x g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

[00373] All AAD-1 (v1) purifications were conducted at 4° C as discussed in Example 10, unless otherwise stated. The cell lysate was loaded onto a Mono Q ion exchange column (Pharmacia Cat. #HR 10/10) equilibrated with 20 mM Tris-HCl (pH 8.0) 1 mM EDTA, followed by 80 mL of washing with the same buffer. The proteins were eluted with 80 mL of a 0 to 0.25 M NaCl linear gradient in column buffer, while 2 mL fractions were collected. The fractions containing AAD-1 were pooled and concentrated using MWCO 30 kDa membrane centrifugation spin columns (Millipore). The sample was then further separated on a Superdex 200 size exclusion column (Pharmacia, XK 16/60) with buffer containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl and 1 mM DTT. Protein concentration was determined by Bradford assay using bovine serum albumin as standard.

[00374] Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 mL of Incomplete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

[00375] 11.1 – Extracting AAD-1 (v3) from plant leaves.

[00376] Approximately 50 to 100 mg of leaf tissue was cut into small pieces and put into microfuge tubes containing 2 stainless steel beads (4.5 mm; Daisy Co., cat. #145462-000) and 300 μL plant extraction buffer (PBS containing 0.1 % Triton X-100 and 10 mM DTT). The tubes were shaken for 4 min with a bead beater at maximum speed followed by centrifugation for 10 min at 5,000x g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

[00377] 11.2 – Bradford assay.

[00378] Total soluble protein concentration from plant leaf tissues were determined by Bradford assay using bovine serum albumin (BSA) as standard. Five micro-liter of serially diluted BSA in PBS or plant extract was transferred to 96-well microtiter plate in triplicates. For standards, concentrations were ranged from 2000 to 15.6 μg/mL. The protein assay concentrate was first diluted 5 fold in PBS and 250 μL was added to each well and incubated at room temp for 5 min. Each optical density (OD) was measured at 595 nm using a microplate reader. The protein concentration of each sample was extrapolated from standard curve using the Softmax® Pro (ver. 4.0) (Molecular Devices).

[00379] 11.3 – Enzyme linked immuno-sorbent assay (ELISA).

[00380] The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-1 antibody (0.5 μg/mL) was coated on 96-well microtiter well and incubated at 4° C for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 μL standard AAD-1 of known concentrations or plant extract (see previous section) was incubated in the wells. For standard curve, purified AAD-1 concentrations ranged from 100 to 1.6 ng/mL in triplicates. Plant extracts were diluted 5, 10, 20, and 40 fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-1 antibody-HRP conjugate (0.25 ug/mL) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 μL 0.4N H_2SO_4. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-1 in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

[00381] For comparison, each sample was normalized with its TSP concentration and percent expression to TSP was calculated.

[00382] 11.4 – Western blotting analysis.

[00383] Plant extracts or AAD-1 standards (5 and 0.5 μg/mL) were incubated with Laemmli sample buffer at 95° C for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane

using standard protocol. After blocking in 4% skim milk in PBS, AAD-1 protein was detected by anti-AAD1 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham cat. #RPN 21058).

Example 12 – Tobacco Transformation

[00384] Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch *et al.*, 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721, *AAD-1 (v3)* + *PAT*), grown overnight in a flask on a shaker set at 250 rpm at 28° C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

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[00385] Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$).

[00386] Prior to propagation, T_0 plants were sampled for DNA analysis to determine the insert copy number. The *PAT* gene which was molecularly linked to *AAD-1 (v3)* was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

[00387] The DNA samples were diluted to 9 ng/µl, then denatured by incubation in a thermocycler at 95° C for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl2 (Third Wave Technologies). An aliquot of 7.5 µl was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well was overlaid with 15 µl of mineral oil (Sigma). The plates were then incubated at 63° C for 1.5 hours and read on the florometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events (**Table 27**).

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Table 27. Tobacco T0 events transformed with pDAB721 (*AAD-1(v3) + PAT*).

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (µg AAD-1 / ml plant extract)	Relative tolerance T0 spray with 2,4-D *
721(1)1	1	+	0.9	Medium
721(2)1	nd	nd	0.6	Medium
721(2)2	5	+	0.3	Low
721(2)3	3	+	2.6	Medium
721(2)5	5	+	4.1	Variable
721(2)6	3	+	0.5	Variable
721(2)8	5	+	0.3	High
721(2)11	3	+	n/a	High
721(2)12	3	+	4.1	Medium
721(2)13	2	+	0.5	Medium
721(2)14	5	+	0.2	High
721(2)16	4	+	3.2	Medium
721(2)17	3	+	nd	High
721(2)18	5	+	nd	High
721(2)19	>10	+	nd	Low
721(2)20	5	+	nd	Medium
721(2)21	4	+	nd	High
721(2)22	7	+	nd	Medium
721(2)23	>10	+	nd	Variable
721(3)003	3	+	nd	Variable
721(3)008	2	+	nd	High
721(3)012	1	+	nd	High
721(3)4	2	+	0.5	High
721(3)5	9	+	3.3	High
721(3)6	4	+	7.1	Variable
721(3)9	2	+	1	Low
721(3)10	3	+	0.6	High
721(3)11	7	+	6	Low
721(3)13	4	+	0.1	High
721(3)014	2	+	0.1	Medium

nd = not done

Legend:	Relative tolerance*	Injury at 3200 g ae/ha 2,4-D (14DAT)
	Low	>50% injury
	Medium	20-50% injury
	High	<20% injury
	Variable	inconsistent

[00388] Copy number estimations were confirmed by Southern Analysis on several events. Southern blot analysis was performed with total DNA obtained from Qiagen DNeasy kit. A total of 2 µgs of DNA was subjected to an overnight digest of NsiI and also HindIII for pDAB721 to obtain integration data. After the overnight digestion an aliquot of ~100 ngs

was run on a 1% gel to ensure complete digestion. After this assurance the samples were processed using same protocol as in Example 6 section 11.

[00389] All events were also assayed for the presence of the *AAD-1 (v3)* gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the Coding Region PCR AAD-1 were (RdpAcodF ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:27) and (RdpAcodR CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C for 3 minutes and 35 cycles of 94° C for 30 seconds, 64° C for 30 seconds, and 72° C for 1 minute and 45 seconds followed by 72° C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 30 PCR positive events were regenerated and moved to the greenhouse.

[00390] A representative plant from each of 19 events was assayed for AAD-1 (v3) expression by ELISA methods previously described. All events assayed showed detectable levels of AAD-1 (v3) (**Table 27**). Protein expression varied across events.

[00391] T_0 plants from each of the 30 events were challenged with a wide range of 2,4-D sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 50, 200, 800, or 3200 g ae/ha to representative clones from each event mixed in deionized water. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 800 g ae/ha or less. Some events were uninjured at this rate (equivalent to 1.5X field rate). All events suffered some level temporary auxinic damage 3 DAT when treated with 3200 g ae/ha. Some leaf burning also occurred at this high rate due to the acidity of the spray solution. Future trials at high 2,4-D rates were buffered. Response of T_0 plants treated with 3200 g ae/ha 2,4-D (~6X field rate) was used to discern relative tolerance of each event into "low" (>50% injury 14 DAT), "medium" (20-50% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable" (**Table 27**).

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[00392] Verification of high 2,4-D tolerance.

[00393] Two to four T_0 individuals surviving high rates of 2,4-D were saved from each event and allowed to self fertilize in the greenhouse to give rise to T_1 seed. Two *AAD-1 (v3)* tobacco lines (event 721(2)-013.010 and 721(3)-008.005) were chosen from the T_0 generation. The T_1 seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4), followed by selective removal of untransformed nulls in this segregating population with 280 g ai/ha glufosinate (PAT selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided medium and high levels of robustness to 2,4-D in the T_0 generation. Improved consistency of response is anticipated in T_1 plants not having come directly from tissue culture. These plants were compared against wildtype KY 160 tobacco. All plants were sprayed with the use of a track sprayer set at 187L/ha. The plants were sprayed from a range of 70-4480 g ae/ha 2,4-D dimethylamine salt (DMA), R-Dichlorprop, and a 50/50 mix of the two herbicides. All applications were formulated in 200mM Hepes buffer (pH 7.5). Each treatment was replicated 4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T_1 generation is segregating, so some variable response is expected due to difference in zygosity. (**Table 28**). No injury was observed at rates below 1X field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by *AAD-1 (v3)*, even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

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Table 28. Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.

Herbicide	KY160 – Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
560 g ae/ha 2,4-D DMA	75	5	0
1120 g ae/ha 2,4-D DMA	80	5	2
2240 g ae/ha 2,4-D DMA	90	5	0
4480 g ae/ha 2,4-D DMA	95	5	5
560 g ae/ha R-dichlorprop	70	5	0
1120 g ae/ha R-dichlorprop	75	5	0
2240 g ae/ha R-dichlorprop	88	5	0
4480 g ae/ha R-dichlorprop	95	10	5
560 g ae/ha 2,4-D DMA/R-dichlorprop	80	5	5
1120 g ae/ha 2,4-D DMA/R-dichlorprop	80	10	10
2240 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15
4480 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15

[00394] A 100 plant progeny test was also conducted on each of the two *AAD-1 (v3)* lines (events 721(2)-013.010 and 721(3)-008.005). The seeds were stratified, sown, and transplanted with respect to the procedure above except null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Both event '013' and '008' segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-1 is heritable as a robust phenoxy auxin resistance gene in multiple species.

[00395] Field level tolerance will be demonstrated by planting T_1 or T_2 seed in the greenhouse, selectively removing the null plants by Liberty selection as previously described, and rearing individual seedlings in 72-well transplant flats (Hummert International) in Metro 360 media according to growing conditions indicated above. Individual plants will be transplanted into the field plots using an industrial vegetable planter. Drip or overhead irrigation will be used to keep plants growing vigorously. Once plants reach 6-12 inches in height, tobacco plants will be sprayed with a broad range of phenoxy auxins and rated as shown above. Environmental stresses are more significant under field conditions; however, based on previous experience with *AAD-1 (v3)*-transformed corn, robust translation of resistance from the greenhouse to the field is expected.

Example 13 – Soybean Transformation

[00396] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00397] Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee *et al.*, 1989; McCabe *et al.*, 1988), cotyledon (Hinchee *et al.*, 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh *et al.*, 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the *AAD-1 (v3)* gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using *AAD-1 (v3)*.

[00398] 13.1 – Transformation Method 1: cotyledonary node transformation of soybean mediated by *Agrobacterium tumefaciens*.

[00399] The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee *et al.*, 1988) and shoot multiplication from apical meristems (McCabe *et al.*, 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee *et al.*, 1988). It remains unclear whether a truly

dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente *et al.*, 2000; Olhoft *et al.*, 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe *et al.*, 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell *et al.*, 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This *de novo* meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee *et al.*, 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00400] 13.1.1 – *Agrobacterium* preparation. The plasmid, pDAB721, contains the *AAD-1 (v3)* gene under the control of the *Arabidopsis* Ubi10 promoter. This plasmid also carries the *PAT* gene under the control of rice actin promoter coding for an enzyme that degrades glufosinate which can be used as a selection agent for transformants. This vector can be used in the transformation experiment described below. The construct pDAB721 was mobilized into the *Agrobacterium* strain EHA101S by electroporation.

[00401] *Agrobacterium* cultures harboring pDAB721 used in the transformations can be grown in YEP medium (10 g/L peptone, 5 g/L yeast extract and 5 g/L NaCl, pH 7.0). *Agrobacterium* cultures are pelleted at low speed and resuspended in SCM liquid medium (see below) to OD660 of 0.6 for use in the inoculations.

[00402] 13.1.2 – Plant transformation. Seeds of "Thorne," "Williams82," or "NE3001," public genotypes of soybean, can be disinfected by a 20-minute wash in 20% (v/v) commercial bleach (NaClO) amended with 2 drops of Liqui-Nox®. The seeds should be rinsed five times with sterile water on hormone-free SHGA medium and allowed to

germinate for 5 days at 24° C, with an 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma cat. #A 8678). Alternatively, certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95 ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added dropwise along the side of the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.*, 1988).

[00403] Explants should be inoculated for 30 minutes. Cocultivation and *Agrobacterium* re-suspension medium consists of B5 medium supplemented with 1 mg/L BAP, 1 mg/L GA3, 3% (w/v) sucrose, 20 mM MES(2-[N-morpholino]ethane sulfonic acid), 400 mg/L L-cysteine (Olhoft and Somers, 2001) pH 5.7, 0.99 mM dithiothreitol (DTT), and 200 μM acetosyringone (AS). Explants are cocultivated for 5 days at 25° C. Following cocultivation, explants were washed in the cocultivation medium containing 100 mg/L timentin and 200 mg/L cefotaxime, without MES and AS.

[00404] Explants are to be placed on shoot induction medium and transferred every 14 days for a total of 28 days prior to herbicide selection. The shoot induction medium consists of full-strength B5 medium supplemented with 1.7 mg/L BAP, 100 mg/L timentin , 200 mg/L cefotaxime pH 5.7 and 3% (w/v) sucrose. Cotyledons should be placed adaxial side up with the cotyledonary nodal region flush to the medium, amended with increasing levels of Basta (2, 5, 6 then 7 mg/L glufosinate ammonium) or sublethal levels of 2,4-D ranging from 10 mg to 400 mg/L every 2 weeks for a total of 8 weeks.

[00405] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from10 mg to 100 mg/L. The elongation medium consisted of B5

medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

[00406] The antibiotics, timentin and cefotaxime, are maintained within the media throughout selection. Cultures are transferred to fresh medium every 2 weeks. Plantlets are acclimated for 1 to 2 weeks prior to transfer to the greenhouse.

[00407] 13.1.3 – Progeny evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1 (v3)* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn. Determination of the inheritance of *AAD-1 (v3)* will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

[00408] 13.2 – Transformation Method 2: "No-shake" *Agrobacterium* mediated transformation of non-regenerable soybean suspension cells.

[00409] The DAS Soybean cell suspensions were cultured on a 3d cycle with 10 ml of settled suspension volume transferred to 115 ml of fresh liquid medium. Settled cell volume was determined by allowing the cell suspension to settle for 2 min in the 125-mL flask after vigorous swirling and then drawing cells from the bottom of the flask with a wide bore 10 ml pipette. The flasks were then transferred to orbital shakers at 140 rpm.

[00410] Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100x25 sterile Petri plate. EHA105 *Agrobacterium* suspension at a density of 1.2 OD^{650} in a 100 μL volume was added and mixed well. The *Agrobacterium* and suspension cell mixture was swirled well and the plate was transferred to dark growth chamber where the temperature was maintained at 25° C.

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[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%

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[00413] 13.3 – Transformation Method 3: aerosol-beam mediated transformation of embryogenic soybean callus tissue.

[00414] Culture of embryogenic soybean callus tissue and subsequent beaming were as described in U.S. Patent No. 6,809,232 (Held *et al.*).

[00415] Embryogenic calluses of several Stine elite varieties, including 96E750, 96E94, 97E986, 96E144 and 96E692, were separately collected into the center of plates of B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K three days after transfer to fresh medium. The tissue was then beamed with pDAB3295 using linearized DNA at a concentration of approximately 0.2 μg/ml. After beaming, the embryogenic callus was transferred to fresh B1-30 3Co5My or B1-30 3Co5My0.25PA0.5K for one passage of a month. The tissue was then transferred to selective medium containing 1 mg/l bialaphos. With bialaphos, selection typically was maintained at 1 mg/l for the first two one-month passages and then increased to 2 mg/l for the following three to seven months. Transgenic events were identified when callus tissue generated by transformation experiments began to organize and develop into embryogenic structures while still on selective media containing 2,4-D plus bialaphos. Once identified, the maturing structures were regenerated into plants according to the following protocol: Embryogenic structures were transferred off B1-30 3Co5My or B1-30 3/co5My0.25PA0.5K to B3 medium. After 3 to 4 weeks' growth on B3 medium, individual structures were transferred to fresh medium. After another 3 to 4 weeks, maturing embryos were transferred to B5G medium and placed in the light. Embryos that had elongated and produced roots were transferred to tubes containing 1/2 B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

[00416] Variations of media referred to in **Table 30** were tested, *e.g.*, B1-30 3Co5My, which was made by adding 3% coconut water and 5 gm/l myo-inositol to B1-30. Other variations included: B1-30 3Co5My0.25 PA0.5K which contained B1-30 basal medium plus 3% coconut water, 5 gm/l myo-inositol, 0.25 gm/l phytic acid, and 0.5 gm/l additional KH_2PO_4 and 1/2 B5G which contained all ingredients of B5G medium at half strength.

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Table 30. Growth Media for Soybean			
	Ingredients in 1 Liter		
	B1-30	**B3**	**B5G**
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		
Activated charcoal		5 g	
Phytagar	8g	8g	
Gelrite			2g
pH	5.8	5.8	5.8

Example 14 -- *AAD-1 (v3)* Enablement in Cotton

[00417] 14.1 – Cotton transformation protocol.

[00418] Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (**Table 31**) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 μE/m2, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

[00419] Cotyledon segments (~5mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (**Table 31**) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (**Table 31**) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (**Table 31**). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

[00420] *Agrobacterium* preparation. Inoculate 35ml of Y media (**Table 31**) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C, while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (**Table 31**) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10^8 colony forming units per mL with a total volume of 40 ml.

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[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.

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Table 31. Media for Cotton Transformation

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

[00423] 14.2 – Experiment specifics.

[00424] For this experiment 500 cotyledon segments were treated with pDAB721. Of the 500 segments treated, 475 had callus isolated while on selection (95% transformation frequency). The callus was selected on glufosinate-ammonium, due to the inclusion of the *PAT* gene in the construct, since there was already a selection scheme developed. Callus line analysis in the form of PCR and Invader were initiated to determine the insertion patterns and to be sure the gene was present at the callus stage, then callus lines that were embryogenic were sent for Western analysis.

[00425] 14.3 – Callus analysis results.

[00426] The object of the analysis is to eliminate any lines that do not have the complete PTU, show no expression or that have high copy number, so those lines are not regenerated. Of the 475 pDAB721 transformed callus lines, 306 were sent for PCR analysis and Invader assay (**Table 32**). Very few lines were PCR negative. The Invader results are not complete at this time, because some samples had low DNA amounts when extracted, and these have been resubmitted. However, the current Invader data shows a few of the lines submitted have high copy number (copy number of >2) (**Table 32**). Due to the large number of lines that passed analysis, it was necessary to decrease the number of embryogenic callus lines being maintained due to the volume. Ninety lines have been sent for Western analysis, and eight of those were negative. The western analysis showed high expression from the majority of the lines (**Table 32**). Eighty-two embryogenic callus lines are being maintained for plant regeneration based on analysis results (and results pending).

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western	Line	Copy #	PTU	Western
1	2	pos	***	43	1	pos	*****
2	1	pos	***	44	low DNA	pos	*****
3	1	pos	***	45	1	pos	*****
4	2	pos	***	46	2	pos	*****
5	1	pos	***	47	1	pos	***
6	1	pos	neg	48	1	pos	***
7	1	pos	****	49	3	faint pos	neg
8	1	pos	*****	50	1	pos	****
9	2	pos	*	51	4	pos	neg
10	1	pos	*	52	2	pos	****
11	1	pos	*****	53	1	pos	***
12	1	pos	*	54	1	pos	****

Table 32. Analysis of cotton callus

Line	Copy #	PTU	Western
13	1	pos	*
14	1	pos	**
15	1	pos	****
16	1	pos	*****
17	1	pos	*
18	1	pos	***
19	1	pos	**
20	1	pos	*****
21	2	pos	*****
22	1	pos	*****
23	1	pos	*****
24	4	pos	* or neg
25	1	pos	****
26	1	pos	****
27	low DNA	pos	*****
28	low DNA	pos	**
29	low DNA	pos	*****
30	17	pos	*
31	low DNA	pos	*****
32	low DNA	pos	*****
33	low DNA	pos	****
34	low DNA	faint pos	*****
35	low DNA	pos	****
36	low DNA	pos	neg
37	low DNA	pos	****
38	low DNA	neg	*****
39	1	pos	****
40	low DNA	pos	*
41	low DNA	pos	*
42	low DNA	pos	**
standard	AAD1	5ug/ml	*****
standard	AAD1	0.5ug/ml	**

Line	Copy #	PTU	Western
55	1	pos	neg
56	5	pos	*
57	2	faint pos	neg
58	8	pos	****
59	2	pos	****
60	5	pos	*****
61	1	pos	**
62	1	pos	**
63	1	pos	***
64	1	pos	***
65	3	pos	****
66	5	pos	*
67	6	pos	*
68	Low DNA	pos	neg
69	Low DNA	pos	****
70	low DNA	pos	**
71	low DNA	faint pos	**
72	low DNA	pos	****
73	low DNA	neg	***
74	low DNA	faint pos	***
75	Low DNA	pos	***
76	Low DNA	pos	neg
77	low DNA	faint pos	****
78	low DNA	pos	****
79	1	pos	**
80	low DNA	pos	***
81	low DNA	pos	***
82	low DNA	pos	***
83	low DNA	neg	****
84	low DNA	pos	***
85	low DNA	pos	**
86	low DNA	pos	*

[00427] 14.4 – Plant regeneration.

[00428] Two *AAD-1 (v3)* cotton lines have produced plants according to the above protocol that have been sent to the greenhouse. To demonstrate the *AAD-1 (v3)* gene provides resistance to 2,4-D in cotton, both the *AAD-1 (v3)* cotton plant and wild-type cotton plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed at 560 g ae/ha 2,4-DMA formulated in 200 mM Hepes buffer (pH 7.5). The plants were evaluated at 3, 7, and 14 days after treatment. Plants were assigned injury ratings with respect to stunting,

chlorosis, and necrosis. Plants assigned an injury rating of 90% or above are considered dead. Three days after treatment (DAT) the wild-type plant began showing epinasy and received a rating of 15%; in contrast, the *AAD-1 (v3)* plant showed 0% injury. By 7 DAT epinasy continued on the wild-type and the new growth shoots began turning brown. It received a rating of 50% at this time. At 14 DAT the *AAD-1 (v3)* plant was still uninjured, whereas the wild-type was severely stunted, and the new growth areas were brown and shriveled. Thus, the wild-type received a rating of 90% at 14 DAT.

[00429] This study demonstrates that the *AAD-1 (v3)* gene in cotton provides substantial tolerance to 2,4-D up to at least 560 g ae/ha.

Example 15 – *Agrobacterium* Transformation of Other Crops

[00430] In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, *see, e.g.,* Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, *see, e.g.,* Hinchee *et al.*, 1988. For *Agrobacterium*-mediated transformation of sorghum, *see, e.g.,* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see, e.g.,* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see, e.g.,* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see, e.g.,* Hiei *et al.*, 1997.

[00431] The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform *AAD-1 (v3)*, for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa*, *perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), Sorghum (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans

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(*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

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(RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

[00436] Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H_2O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C 3.0 minutes heat denature cycle; 95° C 1.0 minute, 50° C 1.0 minute, 72° C 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

[00437] Ten of the resulting white colonies were picked into 3 µl Luria Broth + 1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA GT 3'] (SEQ ID NO:16) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:17) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation *AAD-2 (v1)* for internal consistency.

[00438] 17.2 – Completion of *AAD-2 (v1)* binary vector.

[00439] The *AAD-2 (v1)* gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:18) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:19) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a

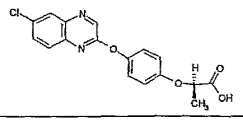
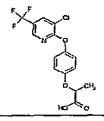
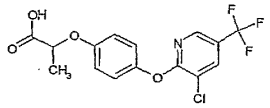
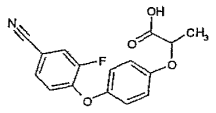
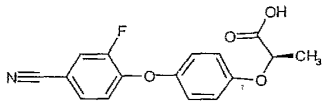
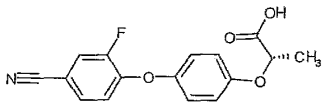
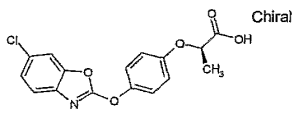
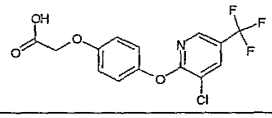
clone with the correct sequence (pDAB716). The AflIII/SacI *AAD-2 (v1)* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2 (v1)*: Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI).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

[00440] 17.3 – Comparison of substrate specificities of AAD-2 (v1) and AAD-1 (v1)

[00441] The activity of an extract from *E. coli* expressing AAD-2 (v1) (pDAB3202) prepared as in Example 11 was tested on four herbicides, 2,4-D, (R,S)-dichlorprop, (R,S)-haloxyfop and (R)-haloxyfop (all at a final concentration of 0.5 mM) using 3 µl (42 µg) of *E. coli* extract per assay with a 15 min assay period. **Figure 22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D = dichlorprop > (R,S)-haloxyfop >> (R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. **Table 33** shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

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Table 33. Comparison of AAD1 and AAD2 activity on various substrates. Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 μM Fe^{2+}, 200 μM Na ascorbate, 1 mM α-ketoglutarate using 4 μl AAD1 (32 μg protein) extract or 3 μl AAD2 extract (42 μg protein).

				A510	
STRUCTURE	**Reg ID**	**Compound**	**Enantiomer**	**AAD1**	**AAD2**
	18706	quizalofop	R	0.27	0.01
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15
	11044492	fenoxaprop	R	0.14	0
	43865	haloxyfop-acetate	--	0	0.22

[00442] The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. *See* **Figure 19.** The K_m values for 2,4-D were 97 and 423 μM for AAD-1 (v1) and AAD-2 (v1) respectively and the apparent V_{max} values were 0.11 and 0.86 A_{510} units, respectively (**Table 34**). Because equivalent amounts of enzyme were used in the assays (as determined by SDS-PAGE analysis), it can be concluded that the k_{cat} of AAD-2 (v1) for 2,4-D is almost 8-fold higher than AAD-1 (v1) and the k_{cat}/K_m is 2-

fold higher. Thus AAD-2 (v1) is significantly more efficient at cleaving 2,4-D *in vitro* than AAD-1 (v1). This is in surprising contrast to the *in planta* findings reported below, where plants expressing AAD-1 (v1) are significantly better in conferring 2,4-D resistance relative to AAD-2 (v1).

Table 34. Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ±SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes: Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

[00443] 17.4 – Evaluation of transformed *Arabidopsis*.

[00444] Freshly harvested T_1 seed transformed with a native [*AAD-1 (v2)*], plant optimized [*AAD-1 (v3)*], or native *AAD-2 (v1)* gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00445] Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, WA) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet and then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed *PAT* gene).

[00446] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and

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plants moved to the greenhouse (22±5° C, 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)*, *AAD-1 (v2)*,or *AAD-2 (v1)* to provide phenoxy auxin herbicide resistance.

[00447] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of 2,4-D (50-800 g ae/ha).

[00448] Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, MO) mixed in 200 mM Tris buffer (pH 9.0).

17.5 – Results of selection of transformed plants.

The first *Arabidopsis* transformations were conducted using *AAD-1 (v3)*. T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (*PAT* gene), equating to a transformation/ selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see **Table 15** in Example 6.5 above). A small lot of native *AAD-1 (v2)* transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency). Surprisingly, *Arabidopsis* transformations using the native *AAD-2(v1)* gene provided a very low transformation frequency when selected for glufosinate tolerance (*PAT* selectable marker function). Approximately 1.3 million seed have been screened and only 228 glufosinate transformants were recovered, equating to a transformation/selection frequency of 0.018% (*see* **Table 15**). Transformation frequency for native *AAD-2 (v1)* was only 6% of that of native *AAD-1 (v2)*. The native *AAD2 (v1)* gene was subsequently synthetically optimized, cloned, and transformed as pDAB3705, into Arabidopsis using methods previously described (see Example 5). The plant optimized *AAD2 (v2)* (SEQ ID NO:29, which encodes SEQ ID NO:30) yielded a normal T_1 *Arabidopsis* selection frequency using Liberty herbicide of approximately 0.11% (*see* **Table 15**).

[00449] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 16** (in

Example 6.5 above) compares the response of *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* and *AAD-1(v2)* genes to impart 2,4-D resistance to *Arabidopsis* T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 16** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)*, *AAD-1 (v3)*, *AAD-2 (v1)* or *AAD-2 (v2)* versus the wildtype or *PAT/Cry1F* transformed controls.

[00450] Surprisingly, *AAD-2 (v1)* transformants were far less resistant to 2,4-D than either *AAD-1 (v2)* or *AAD-1 (v3)* genes (**Table 16**) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) *AAD-1 (v2)*-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average = 34%), and >73% (11 of 15) *AAD-1 (v3)* T_1 plants were uninjured (population injury average = 14%). *See* **Figure 20**. Tolerance improved slightly for plant-optimized *AAD2 (v2)* versus the native gene; however, comparison of both *AAD-1* and *-2* plant optimized genes indicates a significant advantage for *AAD-1 (v3) in planta* (see **Table 16**).

[00451] These results are unexpected given that *in vitro* comparison of native AAD-1 (v2) and AAD-2 (v1) indicated 2,4-D was better degraded by AAD-2 (v1). AAD-2 (v1) is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection from 2,4-D injury is afforded by this expressed protein. Little correlation exists between expression level noted on the Western blot and the level of injury from 2,4-D on the same plants. *See* **Figure 21**. No large difference was evident in protein expression level (*in planta)* for the native and plant optimized *AAD-2* genes. These data corroborate earlier findings that make the functional expression of AAD-1 (v3) *in planta* for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected .

Example 18 – Preplant Burndown Applications

[00452] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

[00453] Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280 – 1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (*e.g.*, horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (*see* federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With *AAD-1 (v3)* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will

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be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed.

Example 19 – In-Crop Use of Phenoxy Auxins Herbicides in *AAD-1 (v3)* Only Transformed Soybeans, Cotton, and Other Dicot Crops

[00454] *AAD-1 (v3)* can enable the use of phenoxy auxin herbicides (*e.g.*, 2,4-D, dichlorprop, MCPA, *et al.*) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560 – 1120 g ae/ha is used. For a complete weed control system, grass weeds must be controlled. A variety of broad spectrum graminicide herbicides, including but not limited to haloxyfop, quizalofop, fenoxaprop, fluazifop, sethoxydim, and clethodim are currently registered for use in most dicot crops which are naturally tolerant to these herbicides. A combination of quizalofop (20 – 100 g ae/ha) plus 2,4-D (420 – 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)*-transformed dicot crop (*i.e.*, soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (*see* weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00455] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if

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broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

[00456] Further benefits could include tolerance to 2,4-D drift or volatilization as mechanisms for off-site 2,4-D injury to dicot crops; no interval required before planting following 2,4-D application (*see* previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicides) can still be used for the subsequent control of *AAD-1 (v3)*-transformed dicot crop volunteers.

[00457] Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1 (v3)* gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - In-Crop Use of Phenoxy Auxins and AOPP Herbicides in *AAD-1 (v3)* Only Transformed Corn, Rice, and Other Monocot Species

[00458] In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with *AAD-1 (v3)* would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. *AAD-1 (v3)*-transformed monocot crops would, therefore,

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enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560 – 1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, *et al.* could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of *AAD-1 (v3)*-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20 – 100 g ae/ha) plus 2,4-D (420 – 840 g ae/ha) could provide two herbicide modes of action in an *AAD-1 (v3)* transformed monocot crop (*e.g.*, corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00459] An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/*AAD-1 (v3)* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-

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crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

[00460] Those skilled in the art will now also recognize that the above example can be applied to any monocot crop that would be protected by the *AAD-1 (v3)* gene from injury by any AOPP herbicide. One skilled in the art of weed control will now recognize that use of various commercial phenoxy auxin herbicides alone or in combination with any commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 – *AAD-1 (v3)* Stacked With Glyphosate Tolerance Trait in Any Crop

[00461] The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (*e.g.*, wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking *AAD-1 (v3)* with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with*AAD-1 (v3)*, one can selectively

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apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a GT trait are stacked in any monocot or dicot crop species:

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

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. *AAD-1 (v3)* + GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

[00462] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or

independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 22 - *AAD-1 (v3)* Stacked with Glufosinate Tolerance Trait in Any Crop

[00463] Glufosinate tolerance (*PAT or bar*) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (*e.g.*, rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

[00464] By stacking *AAD-1 (v3)* with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and

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broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant grass weed species (e.g., *Echinochloa spp* or *Sorghum spp*) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00465] One skilled in the art of weed control will recognize that use of one or more commercial phenoxy auxin herbicides alone or in combination (sequentially or independently) with one or more commercial AOPP herbicide is enabled by *AAD-1 (v3)* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 23 - *AAD-1 (v3)* Stacked with *AHAS* Trait in Any Crop

[00466] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone

herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking *AAD-1 (v3)* with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with *AAD-1 (v3)*, one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where *AAD-1 (v3)* and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

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

   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

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

   iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

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iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha) + 2,4-D (280-1120 g ae/ha) + quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

[00467] One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxy auxin herbicides, or AOPP herbicide, alone or in multiple combinations, is enabled by *AAD-1 (v3)* transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1 (v3)*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 24 – *AAD-1 (v3)* in Rice

[00468] 24.1 – Media description.

[00469] Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/l Phytagel (Sigma). Embryogenic calli were cultured in 100 x 20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25–26° C, and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang *et al.* 1996).

[00470] Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li *et al.* 1993), but adapted to contain 500 mg/l glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang *et al.* 1998) with the inclusion of 30 g/l sucrose in place of maltose. Osmotic medium (NBO) consisted of NB

medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/l hygromycin B for 3–4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/l 6-benzylaminopurine (BAP), 1 mg/l α-naphthaleneacetic acid (NAA), 5 mg/l abscisic acid (ABA) and 50 mg/l hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/l BAP, 0.5 mg/l NAA, and 50 mg/l hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/l hygromycin B (1/2MSH50).

[00471] 24.2 – Tissue culture development.

[00472] Mature desiccated seeds of *Oryza sativa* L. japonica cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3–5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14–17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8–12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14–21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then

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moved to a growth chamber with a 14-h light period at 30° C and in the dark at 21° C for 2–3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C for one week prior to storage at 4° C.

[00473] 24.3 – Microprojectile bombardment.

[00474] All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50 µl water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB3403, 20 µl spermidine (0.1 M) and 50 µl calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60 µl cold 100% ethanol and 8–9 µl was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang *et al.* (1996).

[00475] 24.4 – Tolerance testing.

[00476] Rice plantlets at the 3-5 leaf stage were sprayed with a 0.3% (v/v) solution of DuPont™ Assure® II containing 1% (v/v) Agridex crop oil concentrate using a DeVilbiss bulb sprayer (model 15-RD glass atomizer). This concentration corresponds to approximately 140 g ae/ha. Each plant was spayed in a fume hood at a distance of 8-12 inches with 6 full squirts of the sprayer directed so that the entire plant was covered with an equal portion of herbicide. Each squirt delivered approximately 100 µl solution to the plantlet. Once sprayed, plantlets were allowed to dry for one hour before being moved out of the fume hood. Rating for sensitivity or resistance was done at 10-14 days after treatment (DAT) and is shown in **Table 35** below.

Table 35.

Sample Name	**140 g ae/ha quizalofop**
Control	Dead
63-1A	No injury
63-1F	No injury
63-4B	No injury
63-4D	No injury
63-6C	Dead

[00477] 24.5 – Tissue harvesting, DNA isolation and quantification.

[00478] Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

[00479] 24.6 – Southern blot analysis.

[00480] Southern blot analysis was performed with total DNA obtained from the Qiagen DNeasy kit. A total of 2 μg of DNA was subjected to an overnight digest of HindIII for pDAB3403 to obtain integration data. Likewise a total of 2 ug of DNA was subjected to an overnight digest of MfeI to obtain the PTU data. After the overnight digestion an aliquot of ~100 ng was run on a 1% gel to ensure complete digestion. After this assurance the samples were run on a large 0.85% agarose gel overnight at 40 volts. The gel was then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20 x SSC was then set-up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane was then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200 X100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80°C and then stored at 4° C until hybridization. The hybridization template fragment was prepared using coding region PCR using plasmid pDAB3404. A total of 100 ng of total DNA was used as template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward - ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse - GGGCAGGCCTAACTCCACCAA) (SEQ ID NO:32). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute and 45 seconds followed by 72°C for 10 minutes.

[00481] The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60°C step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it

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RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65°C in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in **Table 36.**

Table 36. Southern Results.

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

[00483] Plants 63-1 A and 63-1 F are not the same event; Plants 63-4 A and 63-4 D are the same event. These events have the expected size PTU's, but they are very complex. This Southern blot PTU data correlates with expression data and the spray data. Sample 63-6 C did not have enough DNA present to perform both Integration and PTU southern blots.

[00484] 24.7 – Western data

[00485] Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in **Table 37.**

Table 37.

Lane	Sample Name	TSP (µg/mL)	ELISA [AAD1] (ng/mL)	ELISA Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	-
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	-
7	AAD1 Standard (0.5 µg/mL)				+++
8	AAD1 Standard (5.0 µg/mL)				+++++

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Example 25 – Turf Grass Transformation Procedures

[00486] Genetic transformation, with *AAD-1 (v3)* substituted for the "bar" gene as described below, of creeping bentgrass mediated by Agrobacterium tumefaciens could be achieved through embryogenic callus initiated from seeds (cv. Penn-A-4), as described generally below. *See* "Efficiency of *Agrobacterium tumefaciens*-mediated turfgrass (*Agrostis stolonifera* L) transformation" (Luo *et. al.*, 2004).

[00487] Callus is infected with an *A. tumefaciens* strain (LBA4404) harboring a super-binary vector that contained an herbicide-resistant bar gene driven either by a rice ubiquitin promoter. The overall stable transformation efficiency ranged from 18% to 45%. Southern blot and genetic analysis confirmed transgene integration in the creeping bentgrass genome and normal transmission and stable expression of the transgene in the T_1 generation. All independent transformation events carried one to three copies of the transgene, and a majority (60–65%) contained only a single copy of the foreign gene with no apparent rearrangements.

[00488] 25.1 – Seed preparation for embryogenic callus induction.

[00489] Mature seeds were dehusked with sand paper and surface sterilized in 10% (v/v) Clorox bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween 20 (Polysorbate 20) with vigorous shaking for 90 min. Following rinsing five times in sterile distilled water, the seeds were placed onto callus-induction medium (MS basal salts and vitamins, 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l 3,6-dichloro-*o*-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP) and 2 g/l Phytagel. The pH of the medium was adjusted to 5.7 before autoclaving at 120°C for 20 min).

[00490] 25.2 – Embryogenic callus induction.

[00491] The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryogenic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

[00492] 25.3 – *Agrobacterium* infection and co-cultivation.

[00493] One day before agro-infection, the embryogenic callus was divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 μM acetosyringone. A 10-ul aliquot of Agrobacterium (LBA4404) suspension (OD=1.0 at 660 nm) was then applied to each piece of callus, followed by 3 days of co-cultivation in the dark at 25°C.

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[00494] 25.4 – Resting stage and *Agrobacterium* control.

[00495] For the antibiotic treatment step, the callus was then transferred and cultured for 2 weeks on callus-induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth.

[00496] 25.5 – Selection and potential transgenic colony identification.

[00497] Subsequently, for selection, the callus was moved to callus-induction medium containing 250 mg/l cefotaxime and 10 mg/l phosphinothricin (PPT) for 8 weeks. Antibiotic treatment and the entire selection process were performed at room temperature in the dark. The subculture interval during selection was typically 3 weeks.

[00498] 25.6 – Regeneration of transgenic plants.

[00499] For plant regeneration, the PPT-resistant proliferating callus events are first moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime, PPT or hygromycin. These calli were kept in the dark at room temperature for 1 week and then moved into the light for 2–3 weeks to develop shoots. No Albino plants with PPT selection (Hygromycin if used as a selection agent, produces a high level of albino plants).

[00500] 25.7 – Root induction and transfer to greenhouse.

[00501] Small shoots were then separated and transferred to hormone-free regeneration medium containing PPT and cefotaxime to promote root growth while maintaining selection pressure and suppressing any remaining *Agrobacterium* cells. Plantlets with well-developed roots (3–5 weeks) were then transferred to soil and grown either in the greenhouse or in the field.

[00502] 25.8 – Vernalization and out-crossing of transgenic plants.

[00503] Transgenic plants were maintained out of doors in a containment nursery (3-6 months) until the winter solstice in December. The vernalized plants were then transferred to the greenhouse and kept at 25°C under a 16/8 h photoperiod and surrounded by non-transgenic wild-type plants that physically isolated them from other pollen sources. The plants started flowering 3-4 weeks after being moved back into the greenhouse. They were out-crossed with the pollen from the surrounding wild type plants. The seeds collected from each individual transgenic plant were germinated in soil at 25°C, and T1 plants were grown in the greenhouse for further analysis.

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[00504] 25.9 – Other target grasses.

[00505] Other grasses that can be targeted for AAD-1 transformation according to the subject invention include Annual meadowgrass (*Poa annua*), Bahiagrass, Bentgrass, Bermudagrass, Bluegrass, Bluestems, Bromegrass, Browntop bent (*Agrostis capillaries*), Buffalograss, Canary Grass, Carpetgrass, Centipedegrass, Chewings fescue (*Festuca rubra commutate*), Crabgrass, Creeping bent (*Agrostis stolonifera*), Crested hairgrass (*Koeleria macrantha*), Dallisgrass, Fescue, Festolium, Hard/sheeps fescue (*Festuca ovina*), Gramagrass, Indiangrass, Johnsongrass, Lovegrass, mixes (Equine, Pasture, etc.), Native Grasses, Orchardgrass, Perennial ryegrass (*Lolium perenne*), Redtop, Rescuegrass, annual and perennial Ryegrass, Slender creeping red fescue (*Festuca rubra trichophylla*), Smooth-stalked meadowgrass (*Poa pratensis*), St. Augustine, Strong creeping red fescue (*Festuca rubra rubra*), Sudangrass, Switchgrass, Tall fescue (*Festuca arundinacea*), Timothy, Tufted hairgrass (*Deschampsia caespitosa*), Turfgrasses, Wheatgrass, and Zoysiagrass.

Example 26 – *AAD-1 (v3)* in Canola

[00506] 26.1 – Canola transformation.

[00507] The *AAD-1 (v3)* gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera* 710 with *Agrobacterium*-mediated transformation. The construct contained *AAD-1 (v3)* gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter.

[00508] Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C, and a photoperiod of 16 hrs light/8 hrs dark.

[00509] Hypocotyl segments (3-5 mm) were excised from 5 - 7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/l kinetin and 1 mg/l 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB721. The *Agrobacterium* was grown overnight at 28° C in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

[00510] After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the

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segments were placed K1D1TC (callus induction medium containing 250 mg/l Carbenicillin and 300 mg/l Timentin) for one week of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/l Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

[00511] Callus samples from 35 independent events were tested by PCR. All the 35 samples tested positive for the presence of *AAD-1 (v3)*,whereas the non-transformed controls were negative (section on PCR assay). Ten callus samples were confirmed to express the AAD-1 protein as determined by ELISA (section on protein analysis).

[00512] Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 1 mg/l Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/l benzylamino purine, 1 mg/l Zeatin, 0.5 gm/l MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/l silver nitrate, 3 mg/l Herbiace, Carbenicillin and Timentin) for another 3 weeks.

[00513] Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH10 (MS, 0.5 gm/l MES, 10 mg/l Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/l Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

[00514] The transformed T0 plants were self-pollinated in the greenhouse to obtain T1 seed. The T0 plants and T1 progeny were sprayed with a range of herbicide concentrations to establish the level of protection by the *AAD-1 (v3)* gene.

[00515] 26.2 – "Molecular analysis": canola materials and methods

[00516] 26.2.1 – Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green

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

[00517] 26.2.2 - Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR *AAD-1 (v3)* were (Forward – ATGGCTCATG CTGCCCTCAGCC) (SEQ ID NO:27) and (Reverse - CGGGCAGGCCTAACTCCACCAA) (SEQ ID NO:28). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 2 minutes followed by 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with *AAD-1 (v3)* events tested positive. Three negative control samples tested negative.

[00518] 26.3 – ELISA.

[00519] Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in **Table 38.**

Table 38. Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (µg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	-
Control 2	61	2549.60	35.00	13.73	-
Control 3	59	2374.41	22.79	9.60	-

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Claims

Claim 1.

A transgenic plant cell comprising a polynucleotide that encodes an AAD-1 protein comprising the amino acid sequence of SEQ ID NO:9 or a variant of SEQ ID NO:9 wherein said variant has aryloxyalkanoate dioxygenase activity, at least one amino acid deletion or conserved substitution, and at least 95% sequence identity with SEQ ID NO:9.

Claim 2.

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

Claim 3.

The cell of claim 2 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

Claim 4.

The cell of claim 2 wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

Claim 5.

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

Claim 6.

The plant of claim 5 wherein said herbicide is a phenoxy auxinic herbicide.

Claim 7.

The plant of claim 5 wherein said herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

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Claim 8.

The plant of claim 5 wherein said herbicide is an aryloxyphenoxypropionate.

Claim 9.

The plant of claim 5 wherein said herbicide is selected from the group consisting of fluazifop, haloxyfop, diclofop, quizalofop, fenoxaprop, metamifop, cyhalofop, and clodinofop.

Claim 10.

The plant of claim 5 wherein expression of said polynucleotide renders said plant resistant to both a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

Claim 11.

The plant of claim 5 wherein said plant further comprises a second herbicide resistance gene.

Claim 12.

The plant of claim 11 wherein said second herbicide resistance gene renders said plant resistant to a herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), and inhibitors of protoporphyrinogen oxidase (PPO).

Claim 13.

A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 5, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and an aryloxyphenoxypropionate herbicide.

Claim 14.

The method of claim 13 wherein said herbicide is an R-enantiomer of a chiral phenoxy auxin.

Claim 15.

The method of claim 14 wherein said herbicide is selected from the group consisting of R-dichlorprop and R-mecoprop.

Claim 16.

The method of claim 13 wherein said herbicide is an achiral phenoxy auxin.

Claim 17.

The method of claim 16 wherein said herbicide is selected from the group consisting of 2,4-D and MCPA.

Claim 18.

The method of claim 13 wherein said first herbicide is an aryloxyphenoxypropionate and said plant is a monocot.

Claim 19.

The method of claim 18 wherein said monocot is selected from the group consisting of corn, rice, wheat, barley, rye, turf grass, oats, sorghum, and pasture grasses.

Claim 20.

The method of claim 13 wherein said first herbicide is a phenoxy auxin and said plant is a dicot.

Claim 21.

The method of claim 20 wherein said dicot is selected from the group consisting of cotton, tobacco, canola, and soybean.

Claim 22.

The method of claim 13 wherein said method comprises applying a second herbicide.

Claim 23.

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

Claim 24.

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

Claim 25.

The method of claim 22 wherein said first herbicide is a phenoxy auxin and second herbicide is an aryloxyphenoxypropionate.

Claim 26.

The method of claim 13 wherein said plant is resistant to glyphosate.

Claim 27.

The method of claim 14 wherein said R-enantiomer is a component of a racemic mixture.

Claim 28.

The method of claim 22 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

Claim 29.

The method of claim 28 wherein said second gene is selected from the group consisting of a modified AHAS (acetohydroxyacid synthase), SurA, SurB, Csr1, Csr1-1, Csr1-2, a modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, GAT, PAT (phosphinothricin-N-acetyltransferase), bar, and a dicamba-degrading enzyme.

Claim 30.

The method of claim 28 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase inhibitors, protoporphyrinogen oxidase inhibitors, and hydroxyphenyl-pyruvate-dioxygenase inhibitors.

Claim 31.

The method of claim 30 wherein said second herbicide is an acetolactate synthase inhibitor selected from the group consisting of imidazolinones, sulfonyl ureas, and triazolopyrimidine herbicides.

Claim 32.

The method of claim 31 wherein said second herbicide is an imidazolinone selected from the group consisting of imazamox, imazethapyr, imazaquin, and imazapic.

Claim 33.

The method of claim 28 wherein said first herbicide is a phenoxy auxin and said second herbicide is selected from the group consisting of glyphosate and glufosinate.

Claim 34.

The method of claim 33 wherein said phenoxy auxin is 2,4-D and said second herbicide is glyphosate.

Claim 35.

The method of claim 28 wherein said first herbicide is an aryloxyphenoxypropionate and said second herbicide is glyphosate.

Claim 36.

The method of claim 35 wherein said first herbicide is selected from the group consisting of quizalofop, haloxyfop, and cyhalofop.

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Claim 37.

The method of claim 31 wherein said second herbicide is a sulfonyl urea selected from the group consisting of amidosulfuron, bensulfuron, chlorimuron, chlorsulfuron, cinosulfuron, flupyrsulfuron, foramsulfuron, halosulfuron, nicosulfuron, primisulfuron, prosulfuron, rimsulfuron, sulfometuron, sulfosulfuron, thifensulfuron, triasulfuron, trifloxysulfuron, and triflusulfuron.

Claim 38.

The method of claim 28 wherein said method further comprises applying a third herbicide.

Claim 39.

The method of claim 38 wherein said herbicides are 2,4-D; quizalofop; and glufosinate.

Claim 40.

A method of controlling weeds in a field, wherein said method comprises applying a first herbicide to said field and planting a seed in said field within 14 days of applying said first herbicide, wherein said seed comprises a cell of claim 1, and wherein said first herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate.

Claim 41.

The method of claim 40 wherein said first herbicide is an acid, an inorganic salt, an organic salt, an ester, an R-enantiospecific isomer, or a component of a racemic mixture.

Claim 42.

The method of claim 40 wherein said seed comprises a second gene that renders said plant resistant to a second herbicide, and said method further comprises applying said second herbicide to said field prior to said planting.

Claim 43.

The method of claim 42 wherein said second herbicide is selected from the group consisting of glyphosate, gramoxone, and glufosinate

Claim 44.

A polynucleotide optimized for expression in a plant wherein said polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

Claim 45.

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

Claim 46.

The polynucleotide of claim 44 wherein said polynucleotide comprises SEQ ID NO:5.

Claim 47.

An isolated polynucleotide that encodes a protein that enzymatically degrades a herbicide selected from the group consisting of a phenoxy auxin and an aryloxyphenoxypropionate, wherein a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, wherein said polynucleotide is operably linked to a promoter that is functional in a plant cell.

Claim 48.

The polynucleotide of claim 47 wherein said promoter is a plant promoter.

Claim 49.

The polynucleotide of claim 47 wherein said promoter is a cassava vein mosaic virus promoter.

Claim 50.

A method of selecting for a transformed plant cell wherein said method comprises subject a plurality of plant cells to transformation with a polynucleotide of claim 44 or 47 and growing said cells in a concentration of a herbicide that permits transformed cells that express said polynucleotide to grow while killing or inhibiting the growth of nontransformed cells, wherein said herbicide is selected from the group consisting of a phenoxy auxin and an aryloxyphenoxyalkanoate.

Claim 51.

The method of claim 50 wherein said cells are cells of a plant and said method is used for selecting a transformed plant.

Claim 52.

A polynucleotide of claim 47 or 50 wherein said polynucleotide is used as a selectable marker in the method of claim 50.

Claim 53.

A method of detecting whether a plant comprises a polynucleotide of claim 47 or 50 wherein said method comprises collecting a sample from said plant and assaying said sample for the presence of said polynucleotide.

Claim 54.

The method of claim 53 wherein said method comprises assaying said sample for the presence of a protein encoded by said polynucleotide.

Claim 55.

The method of 53 wherein said method comprises using a PCR primer or probe to detect for the presence of said polynucleotide.

Claim 56.

The method of 54 wherein said method comprises using an antibody to detect for the presence of said protein.

Claim 57.

A seed comprising a plant cell of claim 1.

Claim 58.

A plant grown from the seed of claim 57.

Claim 59.

A part, progeny, or asexual propagate of the plant of claim 5.

Claim 60.

The method of claim 13 wherein said method is used to treat or prevent herbicide-resistant weeds.

Claim 61.

The plant of claim 5 wherein said plant further comprises an insect-resistance gene derived from an organism selected from the group consisting of *Bacillus thuringiensis, Photorhabdus,* and *Xenorhabdus*.

Claim 62.

The plant of claim 5 wherein said plant further comprises a gene for an agronomic trait selected from the group consisting of fungal resistance, stress tolerance, increased yield, improved oil profile, improved fiber quality, viral resistance, delayed ripening, cold tolerance, and salt tolerance.

Claim 63.

A method of conferring 2,4-D herbicide resistance to a crop wherein said method comprises introducing into at least one plant cell of said crop a nucleic-acid-encoding enzyme having R-specific aryloxyalkanoate dioxygenase activity relative to a 2,4-D herbicide.

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Claim 64.

A method of controlling glyphosate-resistant weeds in a field of glyphosate tolerant crop plants, wherein said plants comprise a polynucleotide of claim 47, and said method comprises applying an aryloxyalkanoate herbicide to at least a portion of said field.

Claim 65.

The method of claim 64 wherein said herbicide is a phenoxy auxin.

Claim 66.

The method of claim 64 wherein said herbicide is an aryloxy phenoxypropionate.

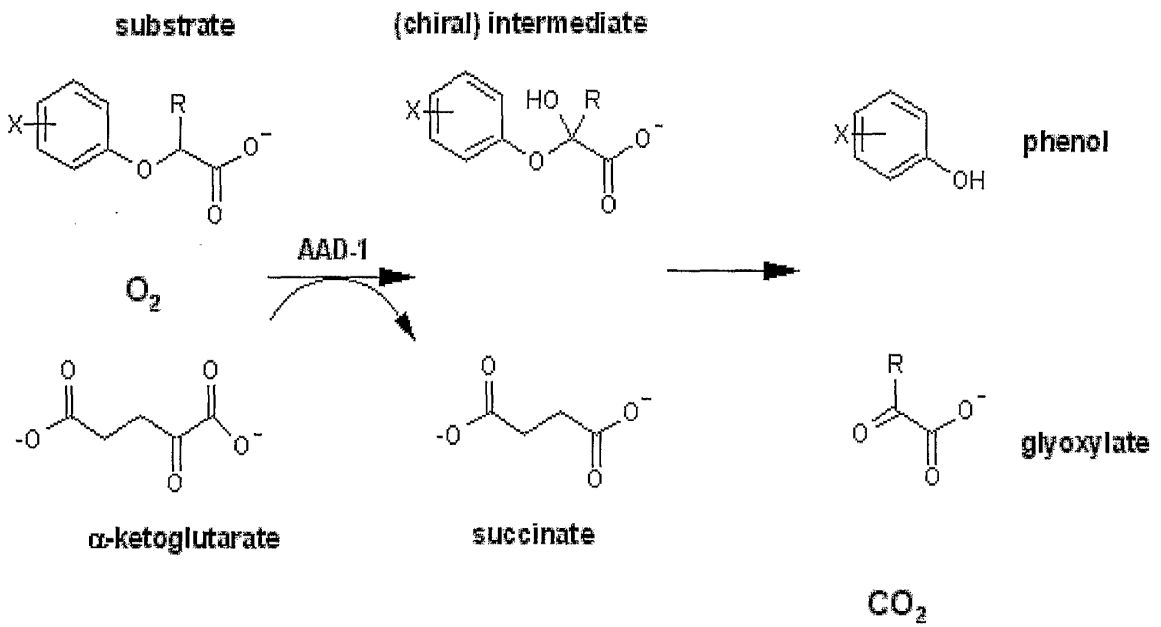
Claim 67.

The method of claim 64 wherein said plants are dicots.

Claim 68.

The method of claim 64 wherein said plants are monocots.

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- Addition of O_2 is *stereospecific*
- Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

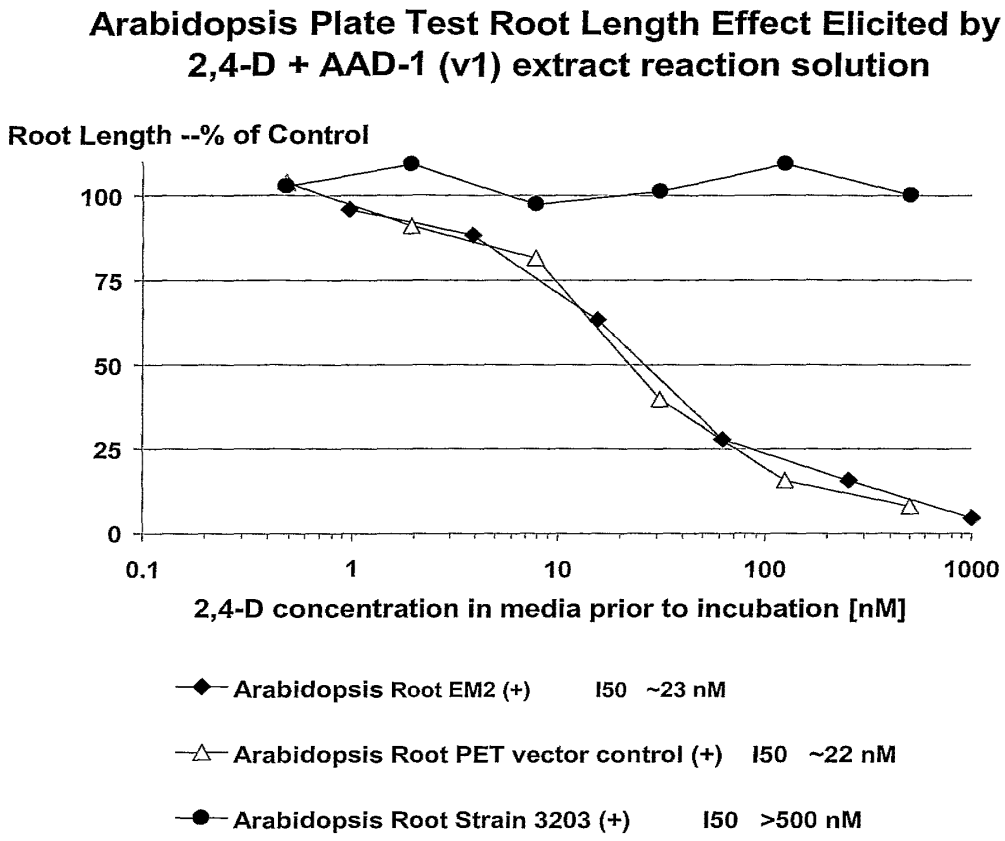

Arabidopsis Plate Test Root Length Effect Elicited by 2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

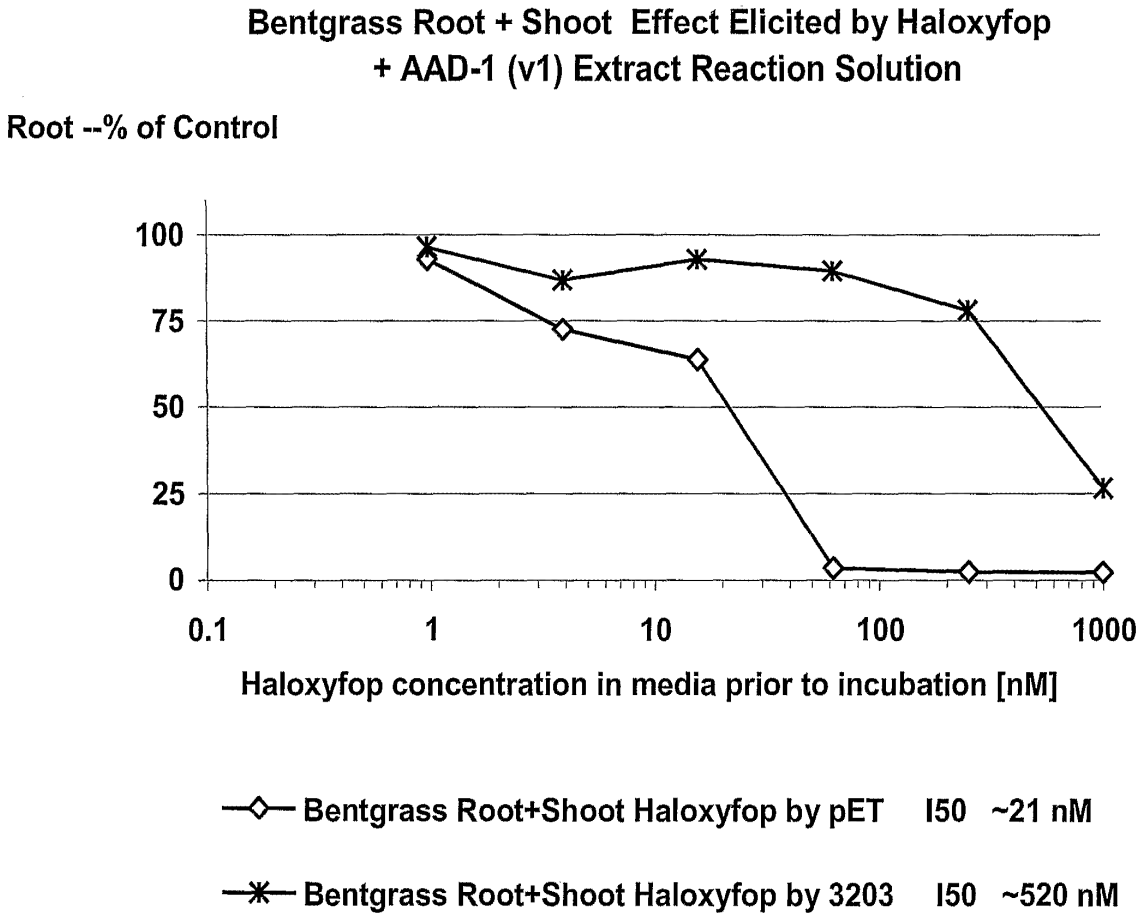

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

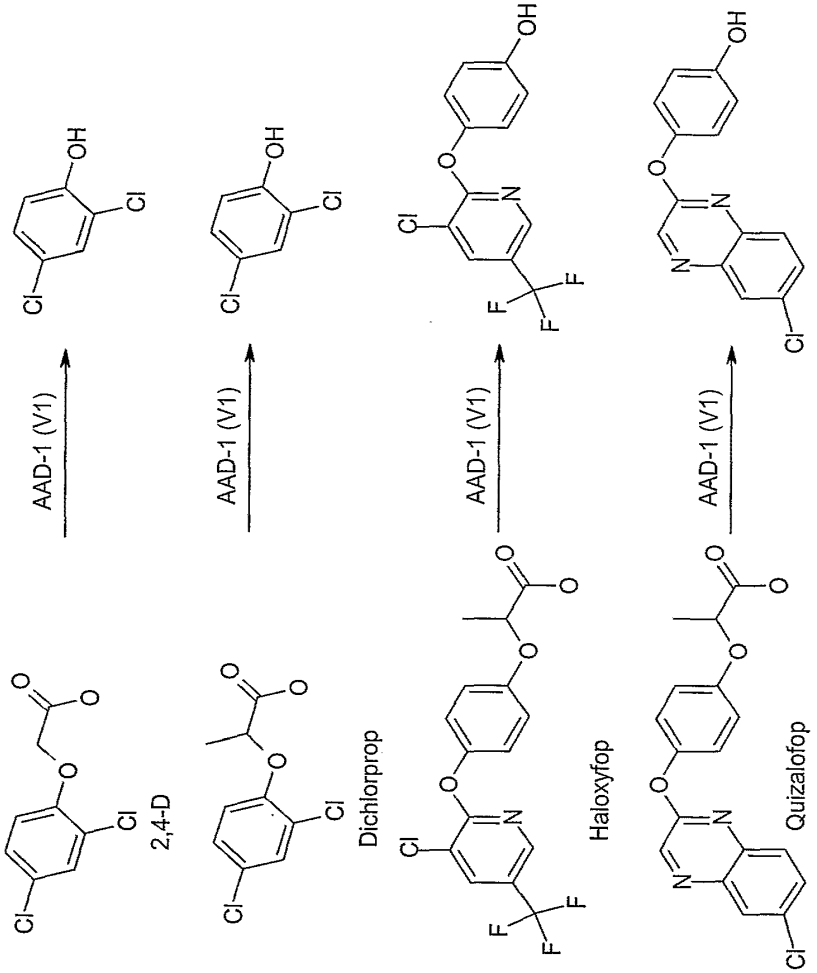

AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
AAD-1 (V1)
2,4-D
Dichlorprop
Haloxyfop
Quizalofop

Fig. 4

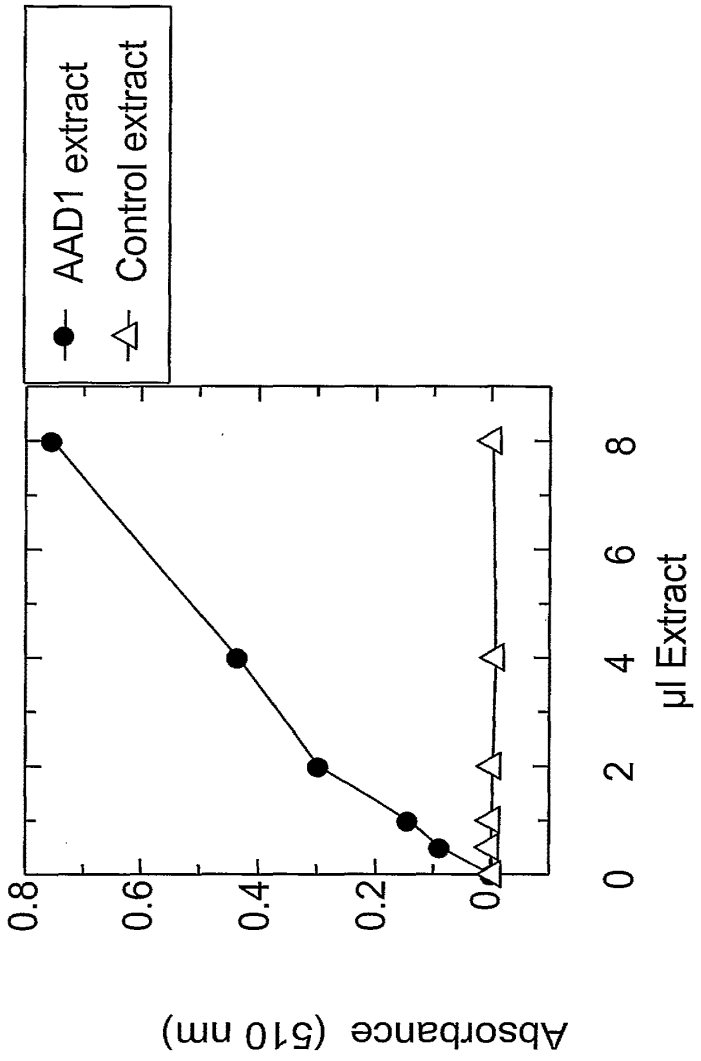

AAD1 extract
Control extract
Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
0
2
4
6
8
µl Extract

Fig. 5

Fig. 6A

Fig. 6B

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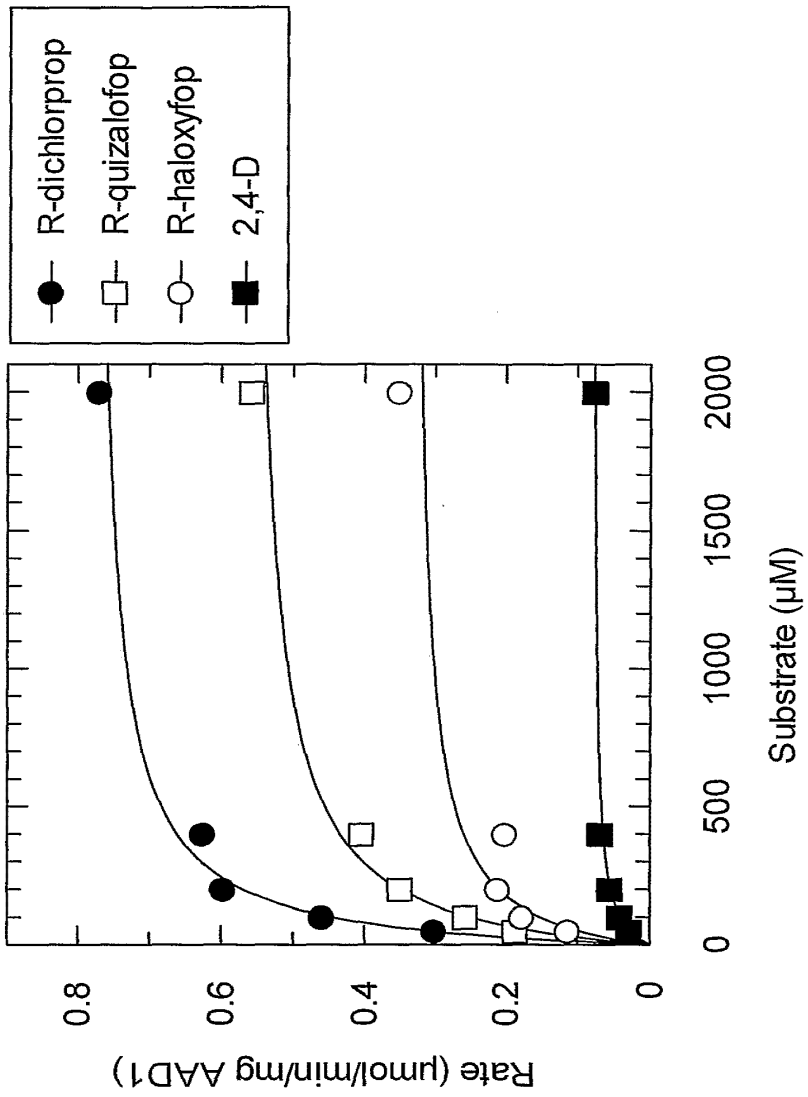

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

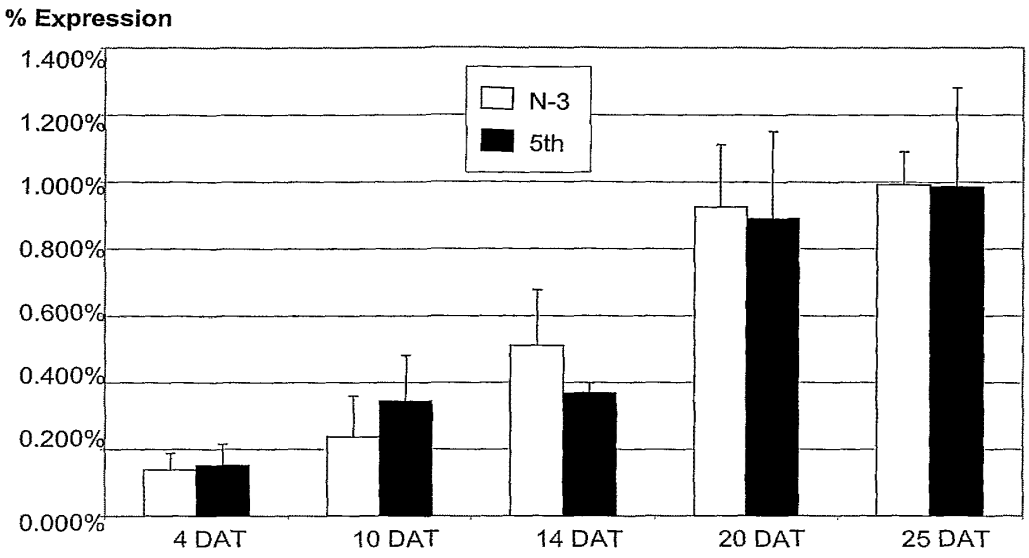

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

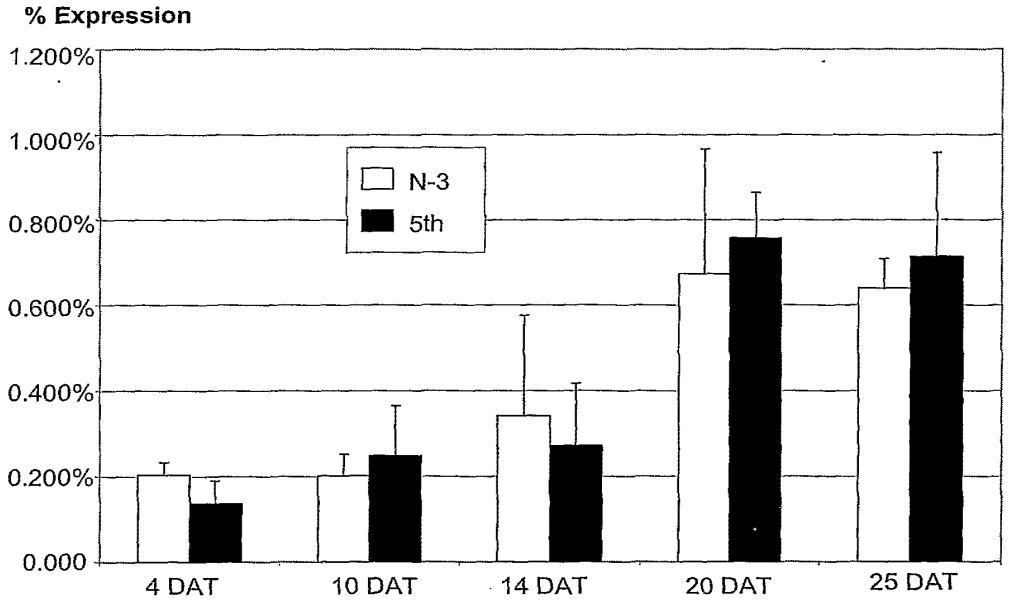

% Expression
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

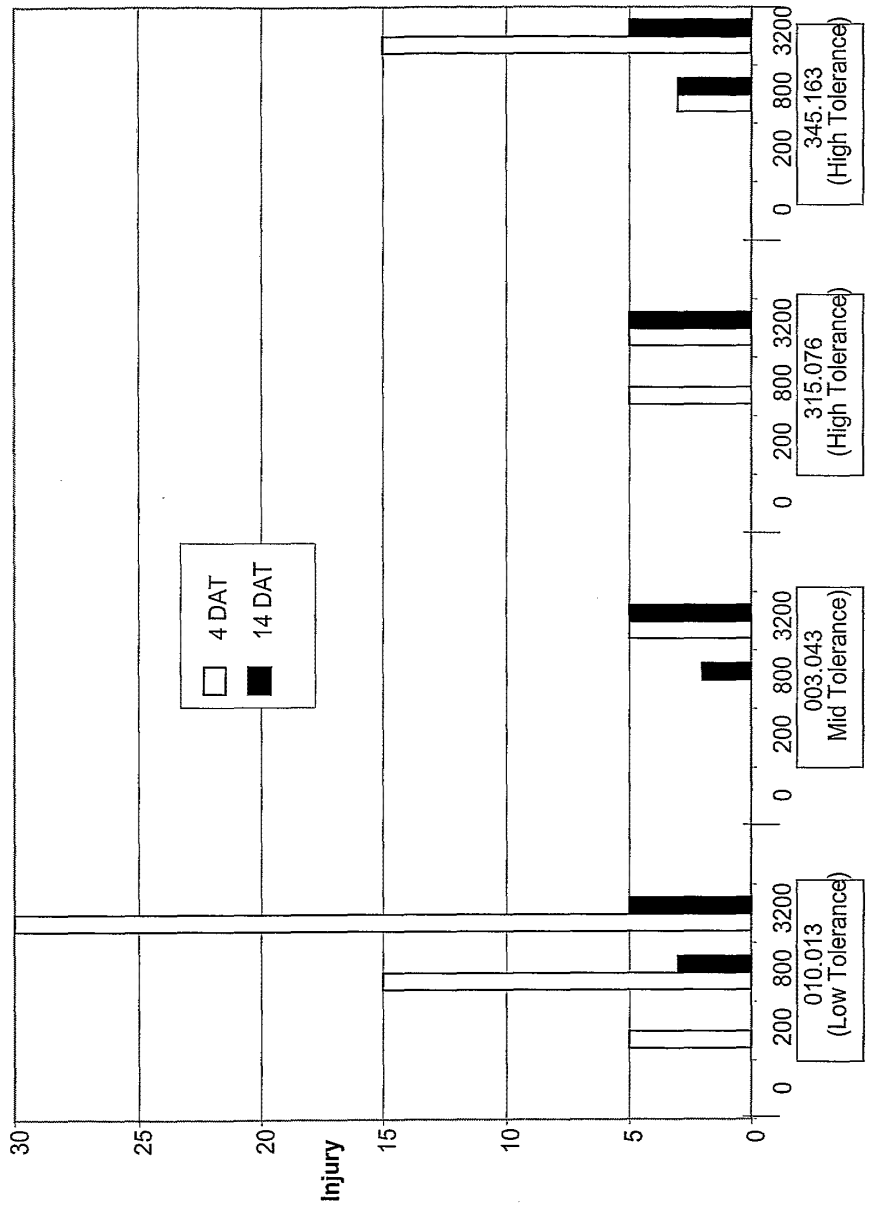


Fig. 9A

Bayer EX1005-0236 (1 of 2)

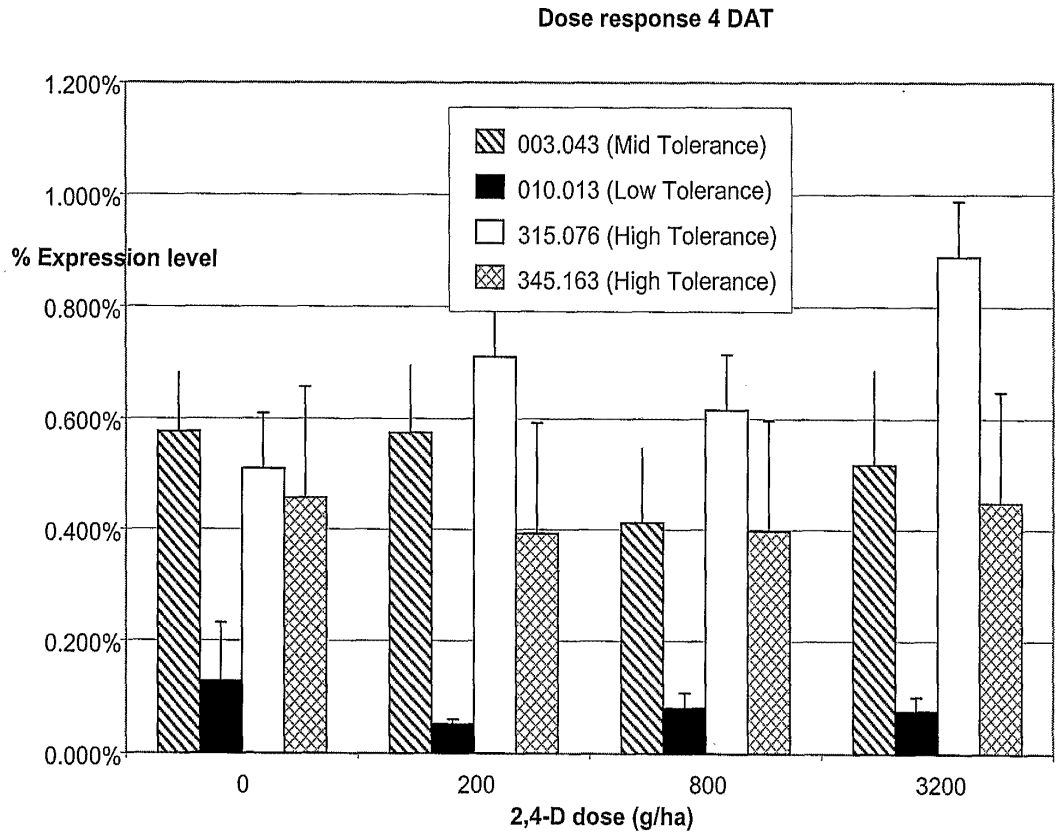

Dose response 4 DAT
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
% Expression level
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

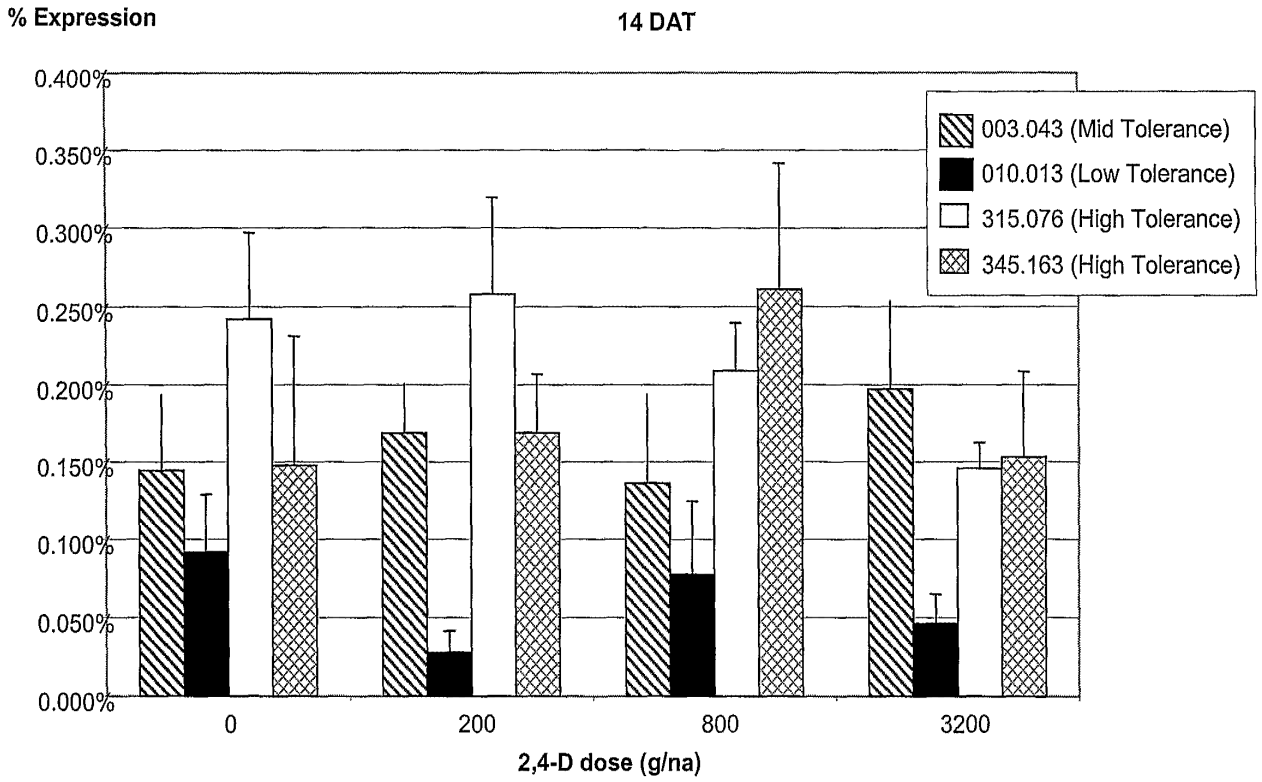

% Expression
14 DAT
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

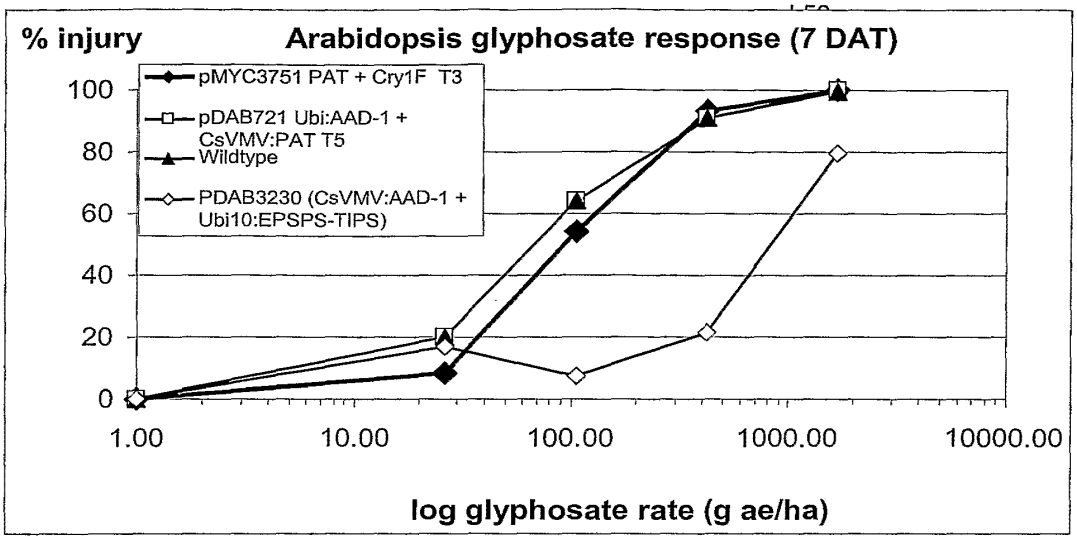

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

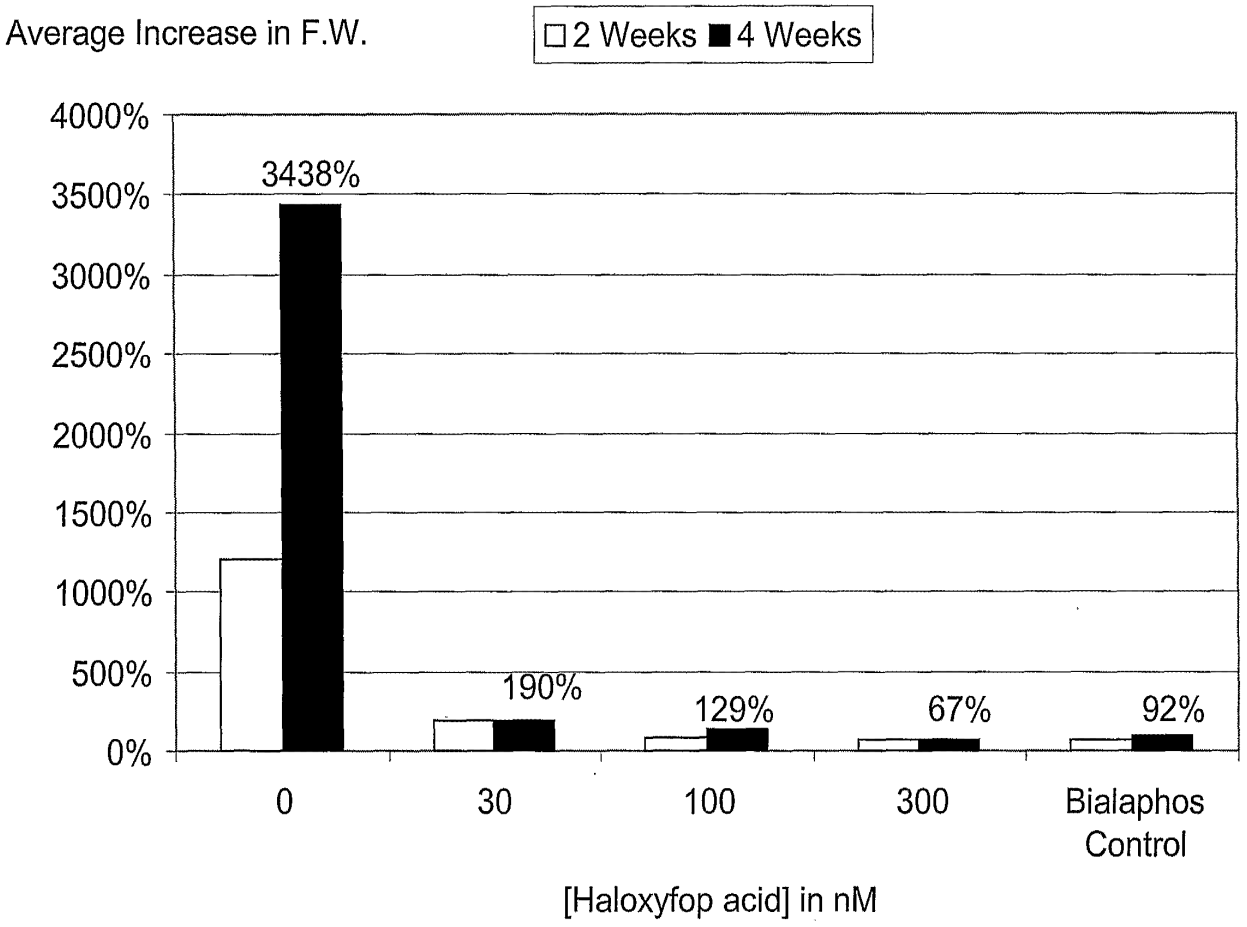

Average Increase in F.W.
2 Weeks
4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

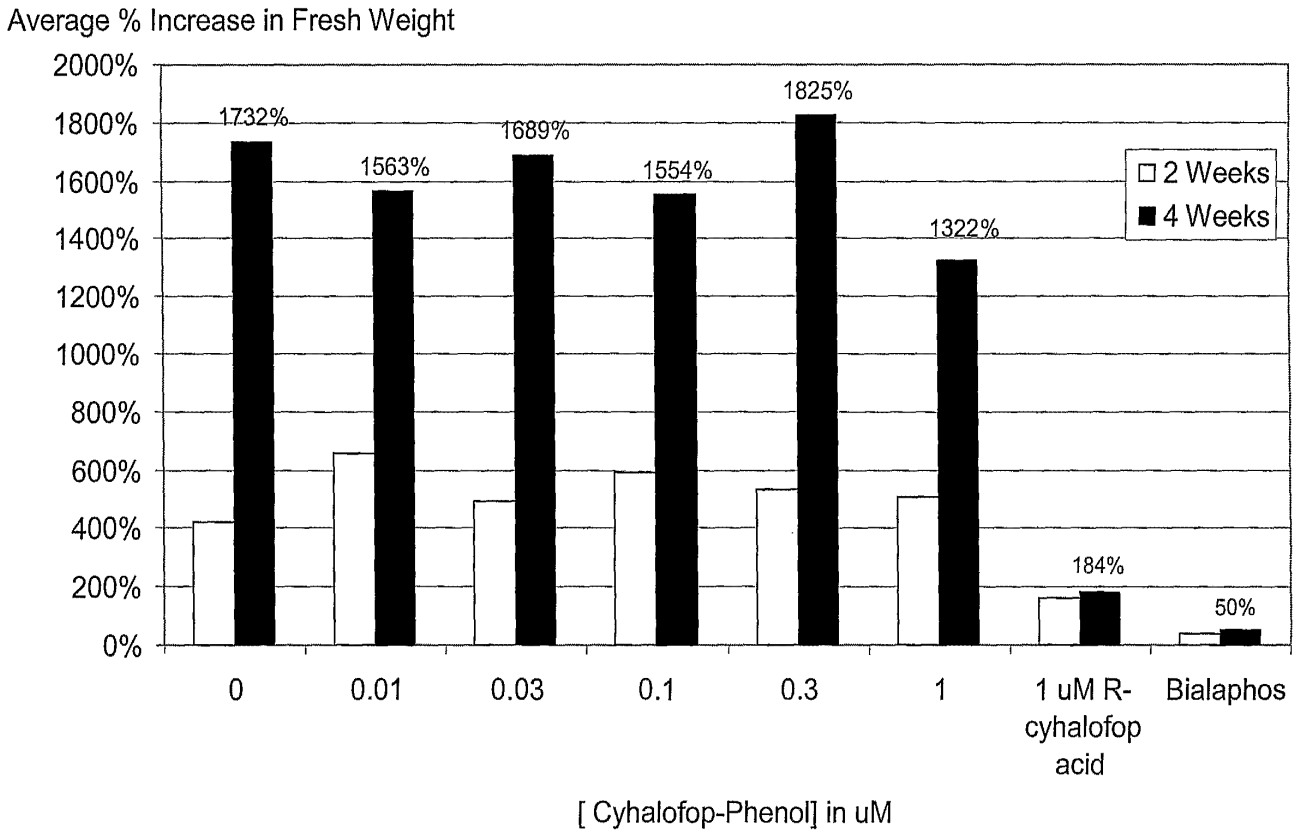

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
2 Weeks
4 Weeks
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

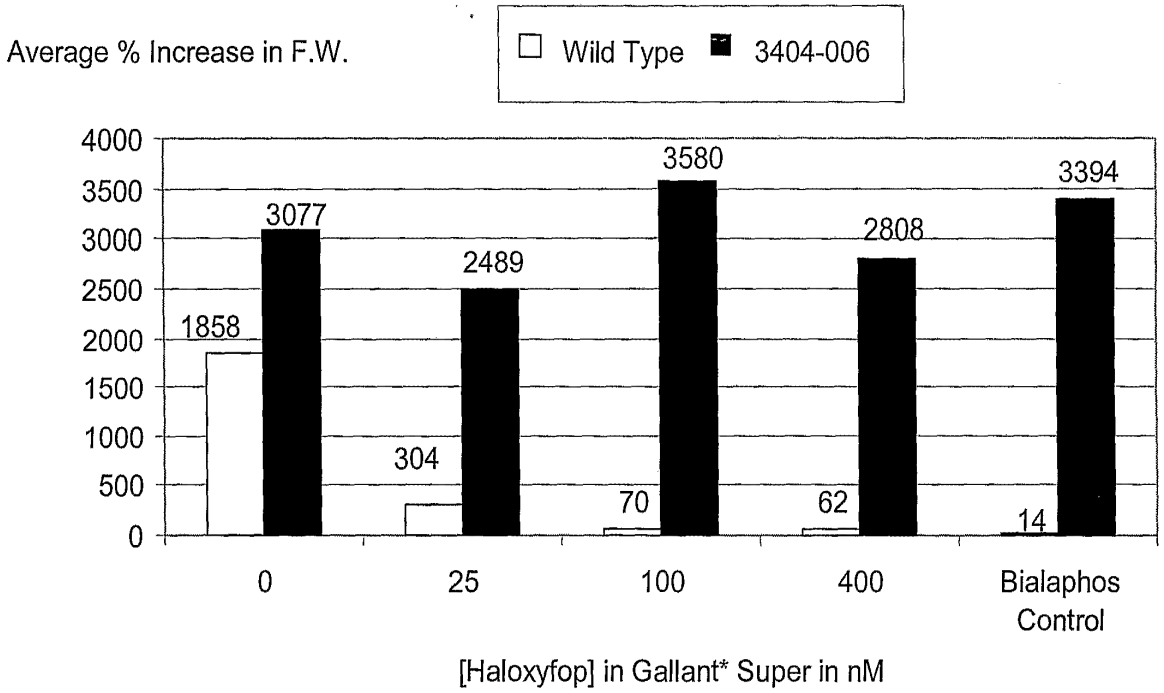

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
1858
3077
304
2489
70
3580
62
2808
14
3394
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant* Super in nM

Fig. 13

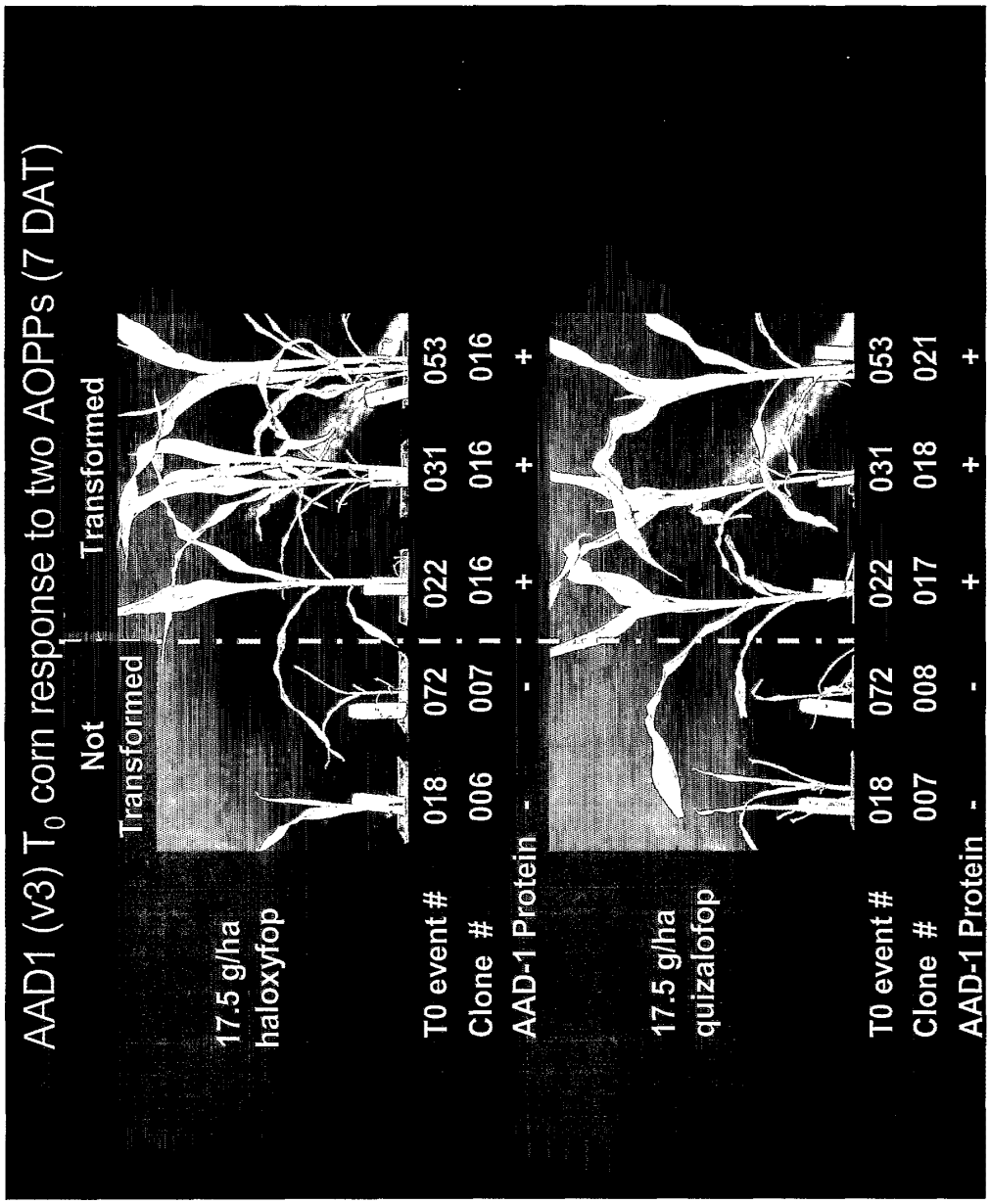

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

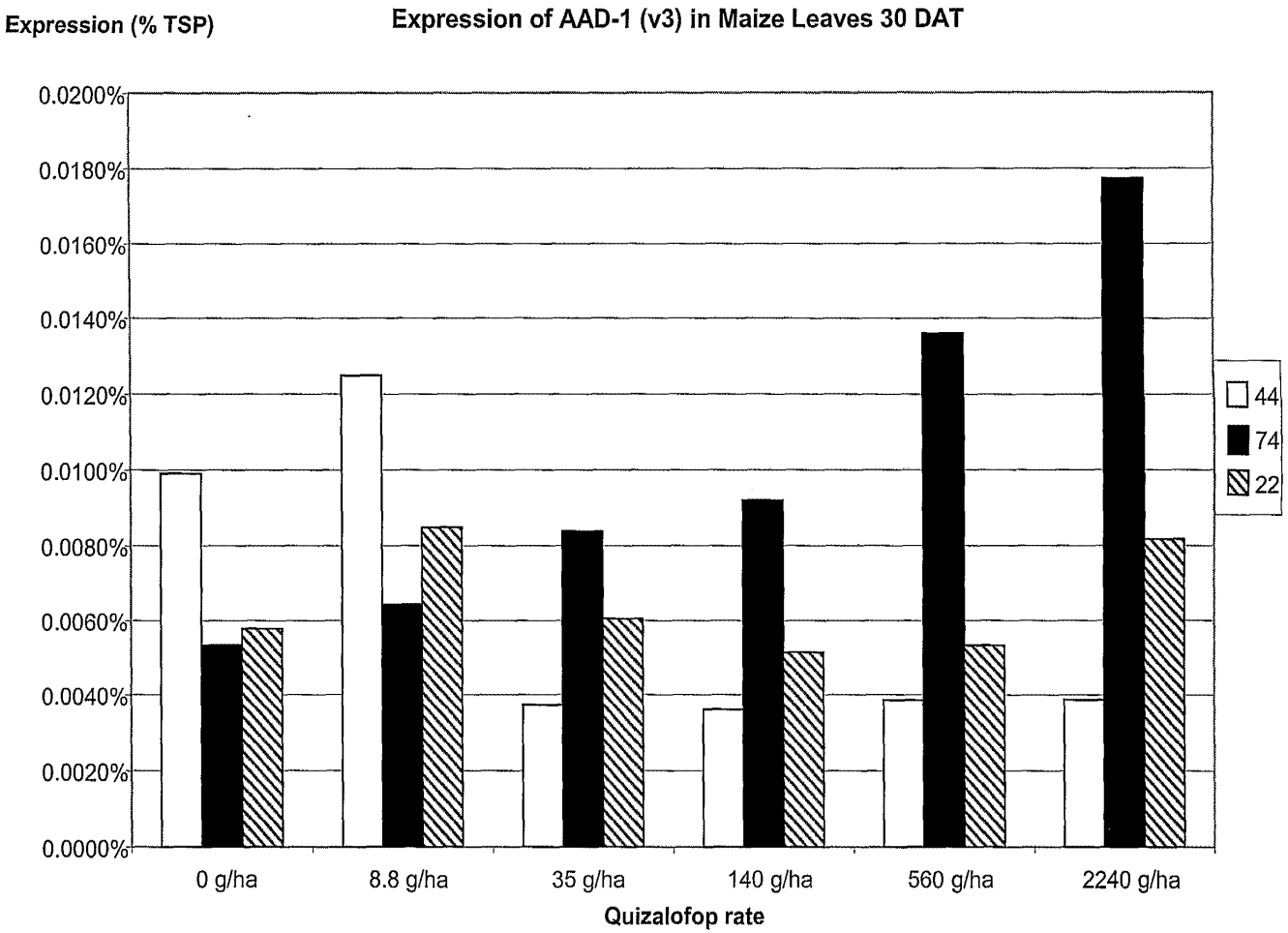

Expression (% TSP)
Expression of AAD-1 (v3) in Maize Leaves 30 DAT
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
44
74
22
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate

Fig. 15

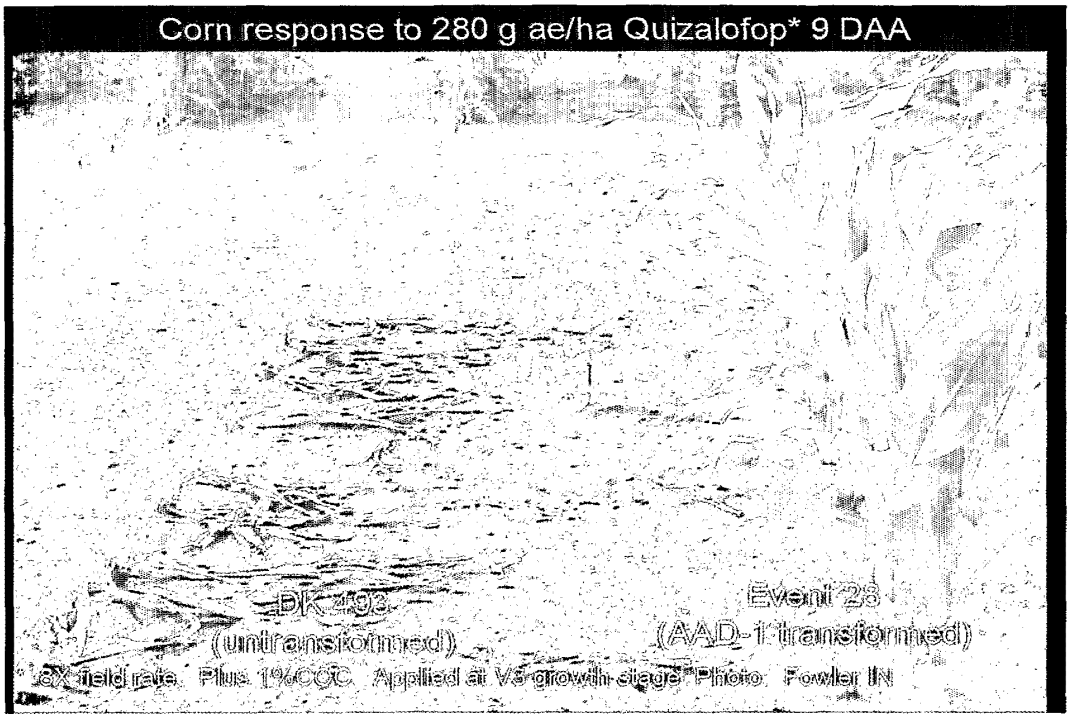

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 493
(untransformed)
Event 28
(AAD-1 transformed)
1X field rate. Plus 1%COC. Applied at V5 growth stage. Photo: Fowler IN

Fig. 16

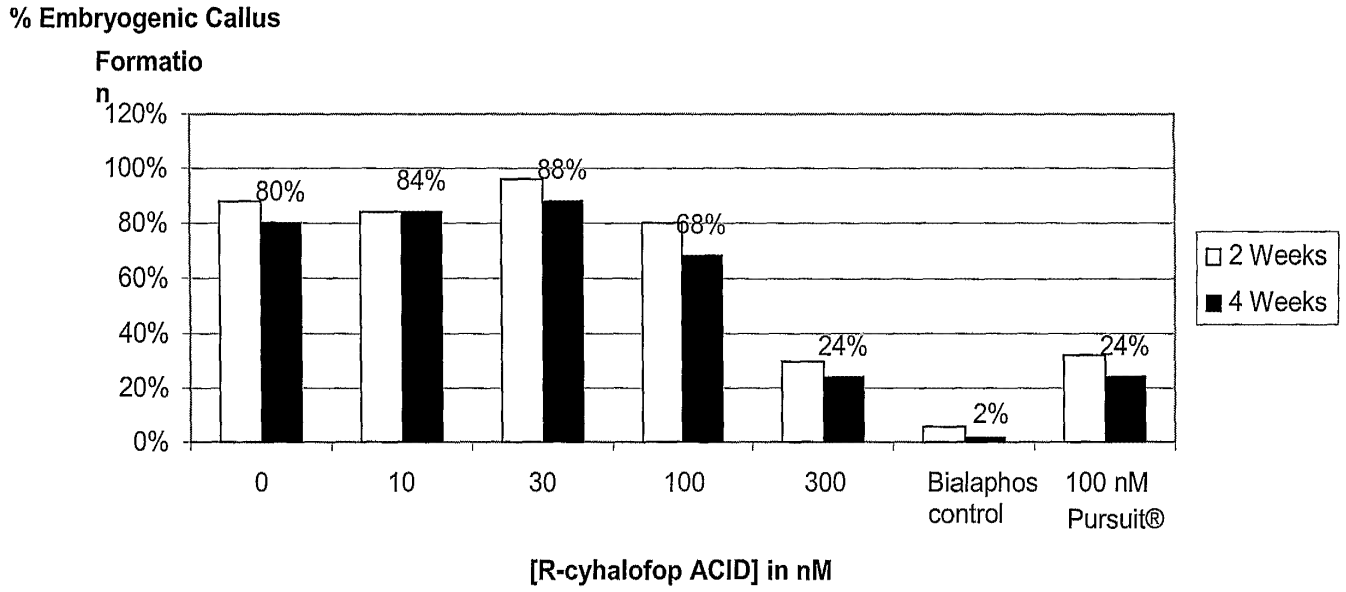
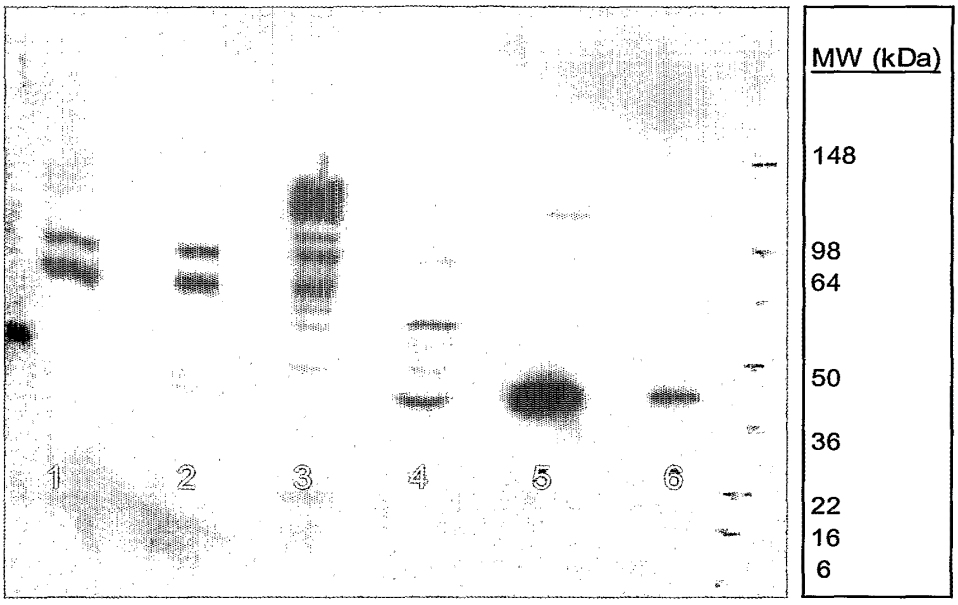

% Embryogenic Callus Formation
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
2 Weeks
4 Weeks
[R-cyhalofop ACID] in nM

Fig. 17



Western	
Lane	
1	Calli #1
2	Calli #2
3	Negative
4	AAD1 Corn
5	Standard 5 ug/mL
6	Standard 0.5 ug/mL

Fig. 18

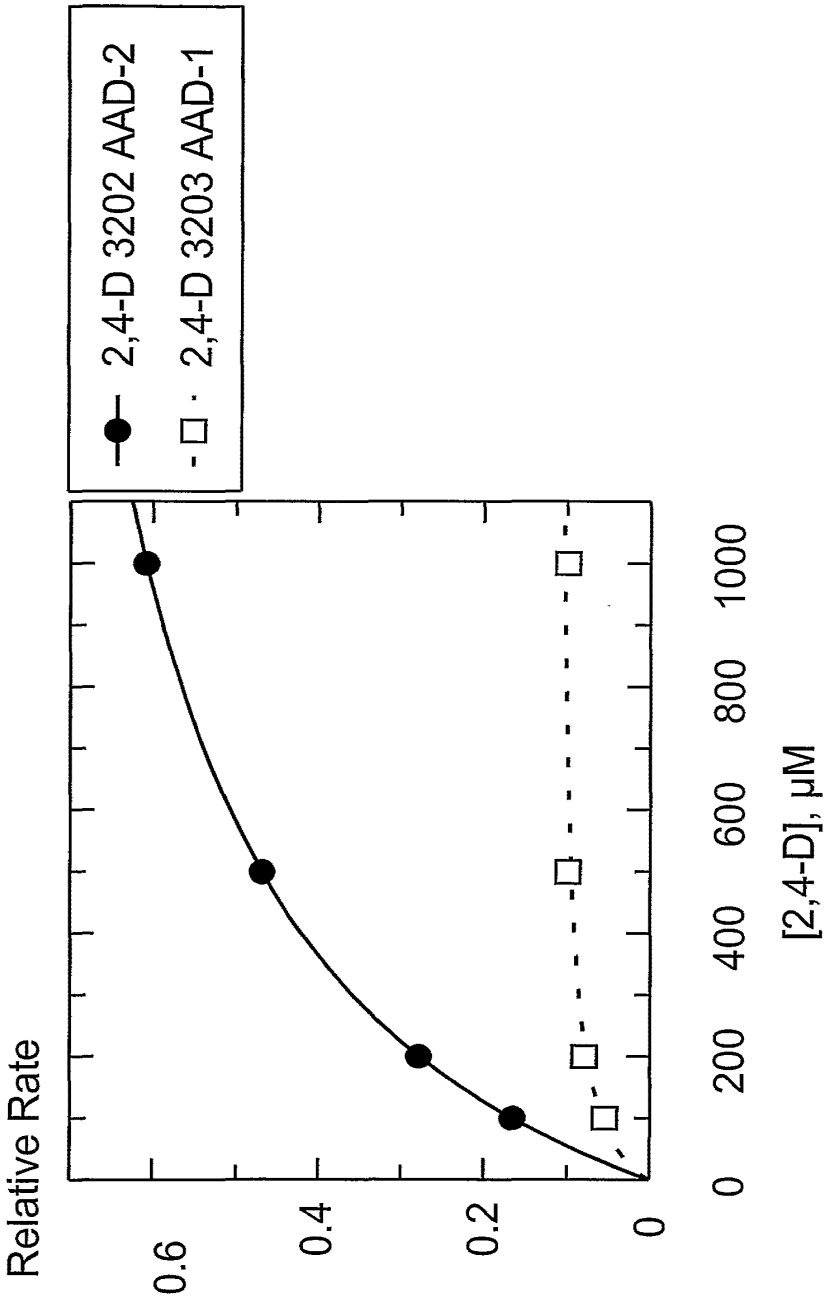

2,4-D 3202 AAD-2
2,4-D 3203 AAD-1
Relative Rate
0.6
0.4
0.2
0
0
200
400
600
800
1000
[2,4-D], µM

Fig. 19

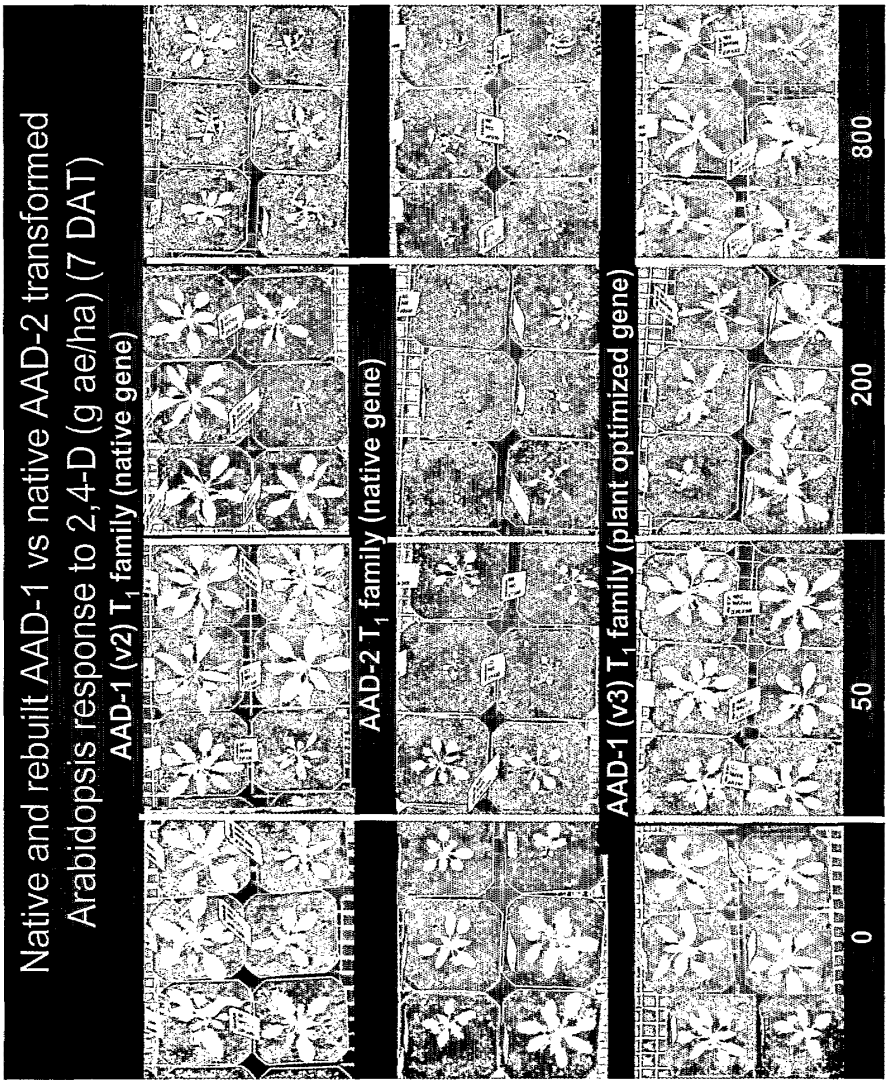

Native and rebuilt AAD-1 vs native AAD-2 transformed Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

Bayer EX1005-0250 (1 of 2)

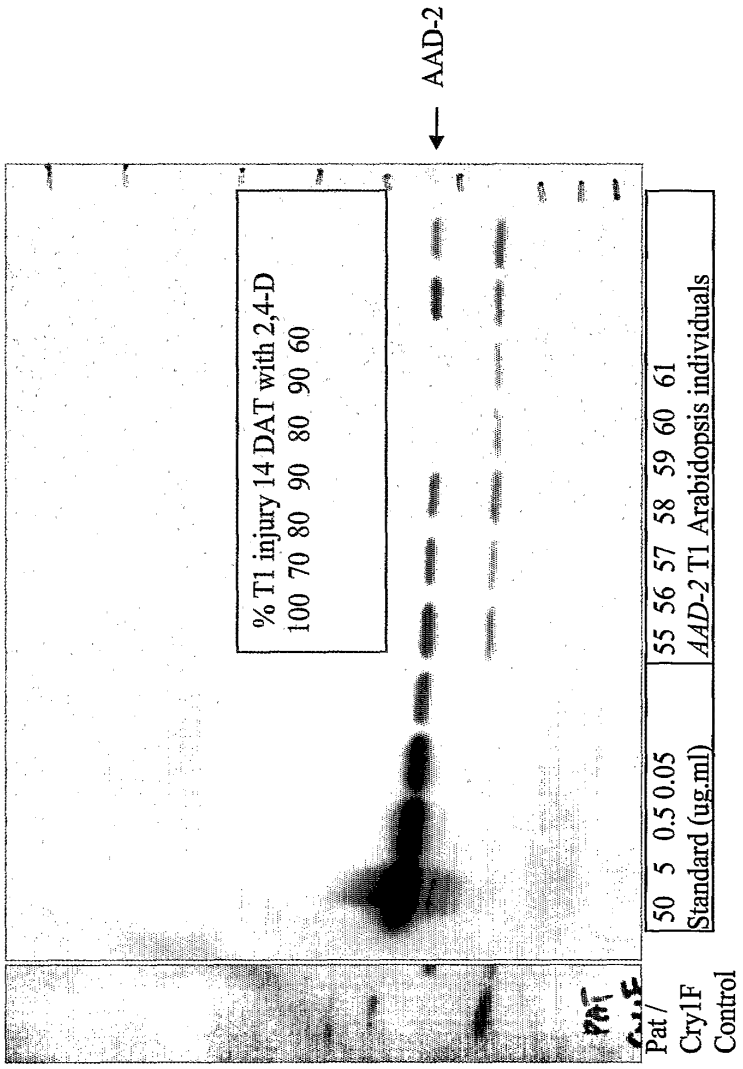


Fig. 21

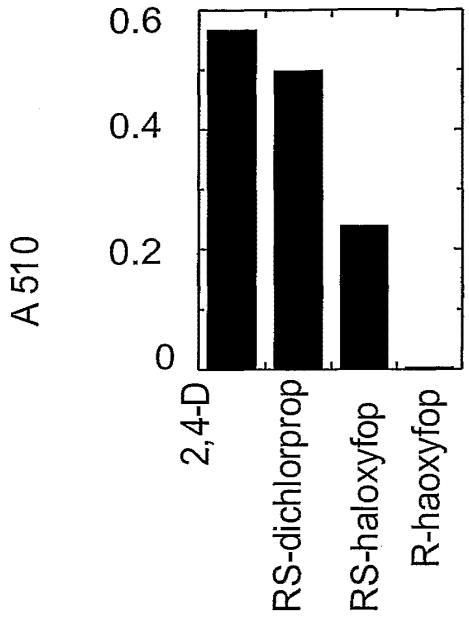


Fig. 22

SEQUENCE LISTING

<110> Dow AgroSciences LLC

<120> Novel Herbicide Resistance Genes

<130> DAS-115XC1

<150> US 60/567,052
<151> 2004-04-30

<160> 32

<170> PatentIn version 3.2

<210> 1
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer used to amplify the rdpA/AAD-1 (v1) gene

<400> 1
tctagaagga gatataccat gcatgctgca ctgtcccccc tctcccagcg 50

<210> 2
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer used to amplify the rdpA/AAD-1 (v1) gene

<400> 2
ctcgagttac tagcgcgccg ggcgcacgcc accgaccg 38

<210> 3
<211> 915
<212> DNA
<213> Sphingobium herbicidovorans

<220>
<221> misc_feature
<222> (1)..(18)
<223> Primer linker

<220>
<221> misc_feature
<222> (907)..(915)
<223> Primer linker

<400> 3
tctagaagga gatataccat gcatgctgca ctgtcccccc tctcccagcg ctttgagcgc 60

atcgcggtcc agccgctgac cggcgtcctg ggcgccgaga tcaccggcgt cgacctgcgc 120

gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttccacac ttaccaggtc 180

atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc 240

ggccccgtcg atcccgtgcc cctgctcaag agcatcgaag ggtatccaga ggtgcagatg 300

atccgccgcg aagccaacga aagcgggcgt gtgatcggtg aygactggca caccgacagc 360

accttcctgg acgcaccgcc ggccgccgtg gtgatgcgcg cgatcgacgt gcccgagcat 420

ggcggcgaca ccggttttct gagcatgtac accgcgtggg agacgctgtc gcccaccatg 480

caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggctcgctc 540

taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg 600

ggcgaccgtg aaaccgtgca ccccctggtg gtgacccatc cgggcagcgg ccgcaagggc 660

ctgtacgtga accaggtcta ttgccagcgc atcgagggca tgaccgatgc cgaaagcaaa 720

ccgctgctgc agttcctgta cgagcatgcg acacggttcg atttcacctg ccgcgtgcgc 780

tggaagaagg accaggtcct ggtctgggac aacctgtgca cgatgcaccg ggccgtaccc 840

gactacgcgg gcaagttccg ctacctgacg cgcaccacgg tcggtggcgt gcgcccggcg 900

cgctagtaac tcgag 915

<210> 4
<211> 897
<212> DNA
<213> Artificial Sequence

<220>
<223> AAD-1 (v2) primary sequence

<220>
<221> misc_feature
<222> (1)..(2)
<223> primer linker

<220>
<221> misc_feature
<222> (891)..(897)
<223> primer linker

<400> 4
ccatggctgc tgcactgtcc cccctctccc agcgctttga gcgcatcgcg gtccagccgc 60

tgaccggcgt cctgggcgcc gagatcaccg gcgtcgacct gcgcgagccg ctcgacgaca 120

gcacctggaa cgaaatcctc gacgcgttcc acacttacca ggtcatctat tttcccggcc 180

aggcgatcac caacgaacag cacatcgcct tcagccggcg cttcggcccc gtcgatcccg 240

tgcccctgct caagagcatc gaagggtatc cagaggtgca gatgatccgc cgcgaagcca 300

acgaaagcgg gcgtgtgatc ggtgatgact ggcacaccga cagcaccttc ctggacgcac 360

cgccggccgc cgtggtgatg cgcgcgatcg acgtgcccga gcatggcggc gacaccggtt 420

ttctgagcat gtacaccgcg tgggagacgc tgtcgcccac catgcaggcc accatcgaag 480

ggttgaacgt agtgcacagc gccacgcgtg tgttcggctc gctctaccag gcccagaacc 540

ggcgcttcag caacaccagc gtcaaggtga tggacgtcga cgcgggcgac cgtgaaaccg 600

tgcacccccc ggtggtgacc catccgggca gcggctgcaa gggcctgtac gtgaaccagg 660

tctattgcca gcgcatcgag ggcatgaccg atgccgaaag caaaccgctg ctgcagttcc 720

tgtacgagca tgcgacacgg ttcgatttca cctgccgcgt gcgctggaag aaggaccagg 780

tcctggtctg ggacaacctg tgcacgatgc accgggccgt acccgactac gcgggcaagt 840

tccgctacct gacgcgcacc acggtcggtg gcgtgcgccc ggcgcgctag tgagctc 897

<210> 5
<211> 919
<212> DNA
<213> Artificial Sequence

<220>
<223> AAD-1 (v3) primary sequence

<220>
<221> misc_feature
<222> (1)..(2)
<223> primer linker

<220>
<221> misc_feature
<222> (6)..(8)
<223> additional alanine codon (GCT)

<220>
<221> misc_feature
<222> (894)..(919)
<223> primer linker

<400> 5
ccatggctca tgctgccctc agccctctct cccaacgctt tgagagaata gctgtccagc 60

cactcactgg tgtccttggt gctgagatca ctggagtgga cttgagggaa ccacttgatg 120

acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcatc tactttcctg 180

gccaagcaat caccaatgag cagcacattg cattctcaag aaggtttgga ccagttgatc 240

cagtgcctct tctcaagagc attgaaggct atccagaggt tcagatgatc cgcagagaag 300

ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttccttgatg 360

cacctccagc tgctgttgtg atgagggcca tagatgttcc tgagcatggc ggagacactg 420

ggttcctttc aatgtacaca gcttgggaga ccttgtctcc aaccatgcaa gccaccatcg 480

aagggctcaa cgttgtgcac tctgccacac gtgtgttcgg ttccctctac caagcacaga 540

accgtcgctt cagcaacacc tcagtcaagg tgatggatgt tgatgctggt gacagagaga 600

cagtccatcc cttggttgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc 660

aagtctactg tcagagaatt gagggcatga cagatgcaga atcaaagcca ttgcttcagt 720

tcctctatga gcatgccacc agatttgact tcacttgccg tgtgaggtgg aagaaagacc 780

aagtccttgt ctgggacaac ttgtgcacca tgcaccgtgc tgttcctgac tatgctggca 840

agttcagata cttgactcgc accacagttg gtggagttag gcctgcccgc tgagtagtta 900

gcttaatcac ctagagctc 919

<210> 6
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> rdpA(ncoI) 5' primer

<400> 6
cccatggctg ctgcactgtc ccccctctcc 30

<210> 7
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> 3'saci primer

<400> 7
gagctcacta gcgcgccggg cgcacgccac cga 33

<210> 8
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> BstEII/ Del NotI 5' primer

<400> 8
tggtggtgac ccatccgggc agcggctgca agggcc 36

<210> 9
<211> 295
<212> PRT
<213> Sphingobium herbicidovorans

<400> 9

Met His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1 5 10 15

Bayer EX1005-0255 (1 of 2)

```
Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
            20                  25                  30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60

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

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110

Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255
```

```
Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295

<210>  10
<211>  295
<212>  PRT
<213>  Artificial Sequence

<220>
<223>  AAD-1 v2 Translation

<220>
<221>  MISC_FEATURE
<222>  (2)..(2)
<223>  Different from v1

<220>
<221>  MISC_FEATURE
<222>  (212)..(212)
<223>  Different from v1

<400>  10

Met Ala Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile Ala
1               5                   10                  15

Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val Asp
            20                  25                  30

Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45

Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60

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

Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95

Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110
```

```
Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125

Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140

Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160

Leu Asn Val Val His Ser Ala Thr Arg Val Phé Gly Ser Leu Tyr Gln
                165                 170                 175

Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205

Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220

Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240

Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255

Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270

Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285

Gly Gly Val Arg Pro Ala Arg
    290                 295
```

<210> 11
<211> 296
<212> PRT
<213> Artificial Sequence

<220>
<223> AAD-1 v3 Translation

<220>
<221> MISC_FEATURE
<222> (2)..(3)
<223> Different from V1

<400> 11

```
Met Ala His Ala Ala Leu Ser Pro Leu Ser Gln Arg Phe Glu Arg Ile
1               5                   10                  15

Ala Val Gln Pro Leu Thr Gly Val Leu Gly Ala Glu Ile Thr Gly Val
            20                  25                  30

Asp Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp
        35                  40                  45

Ala Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr
    50                  55                  60

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

Val Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile
                85                  90                  95

Arg Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His
            100                 105                 110

Thr Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg
        115                 120                 125

Ala Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met
    130                 135                 140

Tyr Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu
145                 150                 155                 160

Gly Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr
                165                 170                 175

Gln Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp
            180                 185                 190

Val Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His
        195                 200                 205

Pro Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln
    210                 215                 220

Arg Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe
225                 230                 235                 240
```

Bayer EX1005-0259 (1 of 2)

Leu Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp
245 250 255

Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
260 265 270

Ala Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr
275 280 285

Val Gly Gly Val Arg Pro Ala Arg
290 295

<210> 12
<211> 888
<212> DNA
<213> Bradyrhizobium japonicum USDA 110

<400> 12
atgacgatcg ccatccggca gcttcagacg cattttgtcg gccaggtttc cggcctcgat 60

ttgcgaaagc cgctcacgcc gggcgaggcc cgcgaggtcg agtccgccat ggacaaatac 120

gcggtgctcg ttttccacga ccaggacatc accgacgagc agcagatggc tttcgcgctg 180

aacttcggcc agcgcgagga cgcgcgcggc ggcacggtca ccaaggagaa ggactaccgg 240

ctgcaatccg gcctgaacga cgtctccaat ctcggcaagg acggcaagcc gctggccaag 300

gacagccgca cgcacctgtt caatctcggc aactgcctct ggcactccga cagctcgttc 360

cgtcccattc ccgcaaaatt ctcgctgctg tcggcgcgcg tggtgaaccc gacgggcggc 420

aacaccgaat tcgcggacat gcgcgccgcc tatgacgcgc tcgacgacga gaccaaggcc 480

gaaatcgagg acctcgtctg cgagcactcg ctgatgtatt cgcgcggctc gctcggcttc 540

accgagtaca ccgacgaaga gaagcagatg ttcaagccgg tcctgcaacg cctcgtgcgc 600

acccatccgg tccaccgccg caagtcgctg tatctctcgt cgcatgccgg caagatcgcc 660

agcatgagcg tgccggaggg gcggctgctg ttgcgcgatc tcaacgagca cgcgacgcag 720

ccggaattcg tctacgtcca caaatggaag ctgcatgacc tcgtgatgtg ggacaaccgc 780

cagaccatgc accgcgtccg ccgctacgac cagtcccagc cccgcgacat gcgccgcgcg 840

acggtggcgg ggacggagcc gacggtgcag cagcaggcgg cggagtag 888

<210> 13
<211> 289
<212> PRT
<213> Bradyrhizobium japonicum USDA 110

<400> 13

Bayer EX1005-0260 (1 of 2)

```
Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val
1               5                   10                  15

Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu
            20                  25                  30

Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln
        35                  40                  45

Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln
    50                  55                  60

Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg
65                  70                  75                  80

Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys
                85                  90                  95

Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys
            100                 105                 110

Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser
        115                 120                 125

Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
    130                 135                 140

Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala
145                 150                 155                 160

Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly
                165                 170                 175

Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys
            180                 185                 190

Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys
        195                 200                 205

Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val
    210                 215                 220

Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln
225                 230                 235                 240
```

Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met
245 250 255

Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln Ser
260 265 270

Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr
275 280 285

Val

<210> 14
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> brjap 5'(speI)

<400> 14
actagtaaca aagaaggaga tataccatga cgat 34

<210> 15
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> br jap 3' (xhoI)

<400> 15
ttctcgagct atcactccgc cgcctgctgc tgc 33

<210> 16
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 forward primer

<400> 16
gtaaaacgac ggccagt 17

<210> 17
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> M13 reverse primer

<400> 17

Bayer EX1005-0262 (1 of 2)

caggaaacag ctatgac 17

<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> NcoI of Brady

<400> 18
tataccacat gtcgatcgcc atccggcagc tt 32

<210> 19
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> SacI of Brady

<400> 19
gagctcctat cactccgccg cctgctgctg cac 33

<210> 20
<211> 525
<212> PRT
<213> Glycine max

<400> 20

Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1 5 10 15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
20 25 30

Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
35 40 45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
50 55 60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65 70 75 80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
85 90 95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
100 105 110

Bayer EX1005-0263 (1 of 2)

```
Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270

Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350
```

```
Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
        435                 440                 445

Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
            500                 505                 510

Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
        515                 520                 525
```

<210> 21
<211> 525
<212> PRT
<213> Artificial sequence

<220>
<223> Doubly mutated Soybean EPSPS protein: threonine 183 converted to isoleucine; proline 187 converted to serine

<400> 21

```
Met Ala Gln Val Ser Arg Val His Asn Leu Ala Gln Ser Thr Gln Ile
1               5                   10                  15

Phe Gly His Ser Ser Asn Ser Asn Lys Leu Lys Ser Val Asn Ser Val
            20                  25                  30
```

Bayer EX1005-0265 (1 of 2)

```
Ser Leu Arg Pro Arg Leu Trp Gly Ala Ser Lys Ser Arg Ile Pro Met
        35                  40                  45

His Lys Asn Gly Ser Phe Met Gly Asn Phe Asn Val Gly Lys Gly Asn
    50                  55                  60

Ser Gly Val Phe Lys Val Ser Ala Ser Val Ala Ala Ala Glu Lys Pro
65                  70                  75                  80

Ser Thr Ser Pro Glu Ile Val Leu Glu Pro Ile Lys Asp Phe Ser Gly
                85                  90                  95

Thr Ile Thr Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu
            100                 105                 110

Leu Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Tyr
        115                 120                 125

Ser Glu Asp Ile His Tyr Met Leu Gly Ala Leu Arg Thr Leu Gly Leu
    130                 135                 140

Arg Val Glu Asp Asp Lys Thr Thr Lys Gln Ala Ile Val Glu Gly Cys
145                 150                 155                 160

Gly Gly Leu Phe Pro Thr Ser Lys Glu Ser Lys Asp Glu Ile Asn Leu
                165                 170                 175

Phe Leu Gly Asn Ala Gly Ile Ala Met Arg Ser Leu Thr Ala Ala Val
            180                 185                 190

Val Ala Ala Gly Gly Asn Ala Ser Tyr Val Leu Asp Gly Val Pro Arg
        195                 200                 205

Met Arg Glu Arg Pro Ile Gly Asp Leu Val Ala Gly Leu Lys Gln Leu
    210                 215                 220

Gly Ala Asp Val Asp Cys Phe Leu Gly Thr Asn Cys Pro Pro Val Arg
225                 230                 235                 240

Val Asn Gly Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly
                245                 250                 255

Ser Val Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu
            260                 265                 270
```

```
Ala Leu Gly Asp Val Glu Ile Glu Ile Val Asp Lys Leu Ile Ser Val
        275                 280                 285

Pro Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Val Ser
    290                 295                 300

Val Glu His Ser Gly Asn Trp Asp Arg Phe Leu Val His Gly Gly Gln
305                 310                 315                 320

Lys Tyr Lys Ser Pro Gly Asn Ala Phe Val Glu Gly Asp Ala Ser Ser
                325                 330                 335

Ala Ser Tyr Leu Leu Ala Gly Ala Ala Ile Thr Gly Gly Thr Ile Thr
            340                 345                 350

Val Asn Gly Cys Gly Thr Ser Ser Leu Gln Gly Asp Val Lys Phe Ala
        355                 360                 365

Glu Val Leu Glu Lys Met Gly Ala Lys Val Thr Trp Ser Glu Asn Ser
    370                 375                 380

Val Thr Val Ser Gly Pro Pro Arg Asp Phe Ser Gly Arg Lys Val Leu
385                 390                 395                 400

Arg Gly Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr
                405                 410                 415

Leu Ala Val Val Ala Leu Phe Ala Asn Gly Pro Thr Ala Ile Arg Asp
            420                 425                 430

Val Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys
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Thr Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr
    450                 455                 460

Cys Val Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Ala Ile Asp Thr
465                 470                 475                 480

Tyr Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Gly
                485                 490                 495

Asp Val Pro Val Thr Ile Lys Asp Pro Gly Cys Thr Arg Lys Thr Phe
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<220>
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gcatccaaga gtcgcatacc aatgcacaag aatgggagtt tcatgggcaa cttcaatgtt 180

gggaaaggca attctggtgt cttcaaagtt tcagcttctg ttgcagccgc agagaaaccc 240

agcacttccc ctgagattgt tcttgaaccc attaaggact tcagtggaac aatcactctg 300

cctggatcaa agagtctttc aaacagaata cttctcttgg cagctctgag tgaaggaacc 360

actgtagttg acaacctttt gtactctgaa gatattcatt acatgttggg tgctctcaga 420

actcttgggt tgagagttga agatgacaag accacaaaac aagccatagt tgaaggatgt 480

ggtgggttgt ttccaacaag caaagaatcc aaagatgaga tcaacttgtt tcttggcaat 540

gctggaattg caatgagaag cctcactgct gcagtagttg cagctggtgg gaatgcaagt 600

tatgtccttg atggtgtccc cagaatgagg gaaaggccca tcggtgacct tgtggctggc 660

ctgaaacagc ttggagcaga tgttgattgc ttcttgggca caaactgccc tccagtgaga 720

gtgaatggga agggaggttt gcctggtgga aaggtcaaac tgagtggatc agtctcttcc 780

cagtatctga ctgccttgct catggctgcc cctctggctt tgggtgatgt ggagattgaa 840

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agtacgcagt tcttgtcttc catgaccaag acatcacaga tgagcaacag atggcctttg 180

ccctcaactt tggtcagagg gaggatgcac gtggtggcac tgtcaccaaa gagaaggatt 240

accgtcttca gtctggcctc aatgatgttt ccaacttggg caaagatgga aagccacttg 300

ccaaggacag ccgcacccat ttgttcaacc ttggaaactg cttgtggcat tctgactcca 360

gcttcagacc aatcccagcc aagttcagcc tcctttctgc tcgtgttgtg aacccaactg 420

gtgggaacac tgagtttgct gacatgagag ctgcctatga tgctcttgac gatgaaacca 480

aagctgagat tgaggacctt gtgtgtgagc actctctcat gtactcaagg ggctcacttg 540

gcttcactga gtacacagat gaagagaagc aaatgttcaa gcccgtcttg cagcgcttgg 600

tccgcacaca ccctgtgcac cgtcgcaaat cactctacct ctccagccat gccggaaaga 660

ttgccagcat gtccgtccct gaagggaggc tccttttgag ggatttgaat gaacatgcta 720

ctcagcctga gttcgtctat gttcacaaat ggaagttgca tgatcttgtg atgtgggaca 780

ataggcaaac catgcacaga gtgaggagat atgaccagtc ccaacccaga gacatgcgcc 840

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gcttaatcac ctagagctcg gtcaccagat ct 932

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20 25 30

Glu Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp
35 40 45

Gln Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly
50 55 60

Gln Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr
65 70 75 80

Arg Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly
85 90 95

Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
100 105 110

Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
115 120 125

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Ser Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu
    130                 135                 140

Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
145                 150                 155                 160

Ala Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg
                165                 170                 175

Gly Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe
            180                 185                 190

Lys Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg
        195                 200                 205

Lys Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser
    210                 215                 220

Val Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr
225                 230                 235                 240

Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
                245                 250                 255

Met Trp Asp Asn Arg Gln Thr Met His Arg Val Arg Arg Tyr Asp Gln
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Thr Val Gln Gln Gln Ala Ala Glu
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Bayer EX1005-0273 (1 of 2)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agents: **SANDERS, Jay, M.** et al.; Saliwanchik, Lloyd & Saliwanchik, A Professional Association, P.O. Box 142950, Gainesville, FL 32614-2950 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(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.

WO 2005/107437 A3

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/14737

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **A01H 1/00**(2006.01),**5/00**(2006.01),**5/10**(2006.01);**C12N 15/52**(2006.01),**15/31**(2006.01),**15/82**(2006.01)
G01N 33/53(2006.01);C12N 9/02(2006.01)

USPC: 536/23.2,23.7;435/419,320.1,468;800/300,298
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/23.2,23.7;435/419,320.1,468;800/300,298

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	NO REFERENCES	

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search
28 February 2006 (28.02.2006)

Date of mailing of the international search report
20 APR 2006

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
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P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (571) 273-3201

Authorized officer
David H. Kruse

Telephone No. (571) 272-0547

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/14737

Continuation of B. FIELDS SEARCHED Item 3:
EAST(USPAT, US-PGPUB, EPO, JPO, Derwent), STN(Agricola, Biosis, CaPlus, Embase), Seqeunce Search of SEQ ID NO: 9

Bayer EX1005-0276 (1 of 2)

Document made available under the Patent Cooperation Treaty (PCT)

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Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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

1359574

THE UNITED STATES OF AMERICA

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APPLICATION NUMBER: *60/567,052*
FILING DATE: *April 30, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/14737*

Certified by

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
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043004

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Express Mail Label No. ER717563543 US

22151 U.S. PTO
60/567052
043004

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Terry	WRIGHT	Indianapolis, IN

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Novel Herbicide Resistance Genes

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☒ Customer Number 23557

OR

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

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

☒ Specification *Number of Pages* 104

☒ Drawing(s) *Number of Sheets* 13

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☒ Other (specify) Tables (16 pages)
Sequences (5 pages)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees.

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 19-0065

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $160.00

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

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Jay M. Sanders

TELEPHONE (352) 375-8100

Date April 30, 2004

REGISTRATION NO. 39,355 *(if appropriate)*

Docket Number: DAS-115P

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

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Docket Number	DAS-115P

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Justin	LIRA	Indianapolis, IN
Don	MERLO	Indianapolis, IN

Number 2 of 2

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Provisional Patent Application
Docket No. DAS-115P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. : DAS-115P

Applicant(s) : Terry Wright, Justin Lira, Donald Merlo

For : Novel Herbicide Resistance Genes

Mail Stop PROVISIONAL PATENT APPLICATION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

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Express Mail No.: ER 717563543 US Date of Deposit: April 30, 2004

ER717563543US

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Lana K. Wilson
Typed/Printed Name of Person Mailing Paper

Signature

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NOVEL HERBICIDE RESISTANCE GENES

Background of the Invention

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

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

[0003] With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More GTCs (*e.g.,* wheat, rice, sugar beets, turf, *etc.*) are poised for introduction pending global market acceptance. 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). (Ng *et al.*, 2003; Simarmata

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et al., 2003; Lorraine-Colwill *et al.*, 2003 ; Sfiligoj, 2004 ; Miller *et al.*, 2003 ; Heap, 2004; 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, 2004); 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, 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 US cotton and soybean acres and >20% of US corn acres (USDA estimates). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium,* and *Commelina* species.

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

[0007] 2,4-D is in the phenoxy acid class of herbicides, as are MCPA 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 an auxin derivative that

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acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known.

[0008] 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).

[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 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.

[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 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 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 many homologues that have a significantly lower homology 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.

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[0012] 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 yet been reported. Literature has focused primarily around close homologues of *tfdA* that primarily degrade achiral phenoxyacetic acids.

[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 phenoxy auxins (such as 2,4-D and dichlorprop), pyridinyloxy auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and dichlofop), 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

[0015] 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

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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-, imidizolanone-, 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] A preferred embodiment of 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, ACCases, 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 (without the *AAD-1* gene) 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 AOPP herbicides, in an enantiospecific manner. No α-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. The preferred enzyme and gene for use according to the subject invention are referred to herein as *AAD-1* (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant HTC trait and selectable marker opportunities.

[0018] 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

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AAD-1 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.

[0019] 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 phenoxyalkanoic auxin herbicides, and aryloxyphenoxyalkanoate 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 a 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** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

[0021] **Figure 2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

[0022] **Figure 3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

[0023] **Figure 4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

[0024] **Figure 5** shows 2,4-dichlorophenol production by recombinant AAD-1.

[0025] **Figure 6** shows phenol production by recombinant AAD-1 from various herbicide substrates.

[0026] **Figure 7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

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[0027] **Figure 8** shows dose response of callused maize suspensions to R-haloxyfop.

[0028] **Figure 9** is a bar graph of the responses of *AAD-1 (v3)*-transformed and non-transformed corn callus to R-haloxyfop.

[0029] **Figure 10** 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.

[0030] **Figure 11** shows fitted curves for 2,4-D degradation rates of AAD-2 vs. AAD-1 (v1).

[0031] **Figure 12** shows dose responses of *AAD-1 v3* (plant optimized), *AAD-1 v2* (native), or *AAD-2* (native)-transformed T_1 *Arabidopsis* to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T_1 family. The relative protective capability from 2,4-D injury of individual genes is plant optimized *AAD-1 (v3)* > native *AAD-1 (v2)* > native *AAD-2*.

[0032] **Figure 13** shows western blot analysis of individual native *AAD-2*-transformed T_1 *Arabidopsis* plants. This shows that plants expressing the AAD-2 protein are suffering severe injury from 200 g ae/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.

Brief Description of the Sequences

[0033] **SEQ ID NO:1** is the sequence of a forward primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0034] **SEQ ID NO:2** is the sequence of a reverse primer used to amplify the *rdpA/AAD-1 (v1)* gene.

[0035] **SEQ ID NO:3** is the nucleotide sequence of *AAD-1 (v1)* from *Sphingobium herbicidovorans*.

[0036] **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 inadvertant change was made at amino acid #212 from arginine to cysteine.

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[0037] **SEQ ID NO:5** is a "plant-optimized" DNA sequence ("*AAD-1 (v3)*"). This "gene" encodes essentially the identical protein (disclosed herein as SEQ ID NO. 10) as the native gene (disclosed herein 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.

[0038] **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).

[0039] **SEQ ID NO:8** is another PCR primer ("BstEII/ Del NotI") that was used with the "3' SacI" primer.

[0040] **SEQ ID NO:9** is the native amino acid sequence encoded by the *AAD-1 (v1)* gene from *Sphingobium herbicidovorans*.

[0041] **SEQ ID NO:10** is the amino acid sequence encoded by the *AAD-1 (v2)* DNA sequence of SEQ ID NO:4.

[0042] **SEQ ID NO:11** is the amino acid sequence encoded by the *AAD-1 (v3)* plant-optimized DNA sequence of SEQ ID NO:5.

[0043] **SEQ ID NO:12** is the DNA sequence of the native *AAD-2* gene.

[0044] **SEQ ID NO:13** is the amino acid sequence of the AAD-2 protein.

[0045] **SEQ ID NO:14** is a forward primer used to amplify *AAD-2* DNA for cloning.

[0046] **SEQ ID NO:15** is a reverse primer used to amplify *AAD-2* DNA for cloning.

[0047] **SEQ ID NO:16** is the M13 forward primer.

[0048] **SEQ ID NO:17** is the M13 reverse primer.

[0049] **SEQ ID NO:18** is a forward primer used to amplify *AAD-2* DNA for cloning.

[0050] **SEQ ID NO:19** is a reverse primer used to amplify *AAD-2* DNA for cloning.

Detailed Description of the Invention

[0051] Development of a 2,4-D resistance gene and subsequent resistant crops could provide an excellent opportunity to control 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

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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.

[0052] An additional benefit of the *AAD-1* gene is its unprecedented ability to concomitantly degrade a whole 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, 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.

[0053] 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 (acid equivalent)/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds.

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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. Combination 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.

[0054] 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).

[0055] It is important to have flexibility in controlling a broad spectra 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

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optimal combination(s) and timing of these application(s) will be situation, species, and environmentally-dependent and best made by a person skilled in the art of weed control.

[0056] 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.

[0057] 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 glufosinate tolerance (*e.g.*, *Pat, bar*), ALS-inhibiting herbicide resistance (*e.g.*, imidazolinone, sulfonylurea, and other chemistries = *AHAS, Csr1, SurA, et al.*), bromoxynil resistance (*e.g.*, *Bxn*), resistance to inhibitors of HPPD enzyme, resistance to inhibitors of phytoene desaturase, resistance to photosystem II inhibiting herbicides (*e.g.*, *psbA*), resistance to photosystem I inhibiting herbicides, resistance to PROTOX-inhibiting herbicides (*e.g.*, Acuron™), resistance to phenylurea herbicides (*e.g.*, *CYP76B1*), 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.

[0058] 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 to provide a complete agronomic package of improved crop quality with the ability to cost effectively and with flexibility control any number of agronomic pests.

[0059] 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

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characterized by three conserved histidine residues and in a "HX(D/E)X_{23-26}(T/S)$X_{114-183}$HX_{10-13}R" motif which comprises the active site. The histidine 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.

[0060] More specifically, the subject invention relates 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.

[0061] 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.

[0062] 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* plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

[0063] 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.

[0064] 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. This highly novel discovery is the basis of significant HTC trait opportunities.

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[0065] The exemplified genes and proteins, and future homologues discovered because of the benefit of the subject disclosure, will provide great benefit to the agricultural community. 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.

[0066] 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 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*.

[0067] *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

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plants to render the plants resistant to 2,4-D (not to mention the completely surprising AOPP resistance).

[0068] 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-ketoglutaratedependent 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.

[0069] 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.

[0070] 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).

[0071] 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 aryloxyalkanoate dioxygenase enzyme (AAD-1). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-1) capable of degrading phenoxy auxin and aryloxyphenoxypropionate herbicides.

[0072] *AAD-1*, and future homologues discovered because of the benefit of the subject disclosure, can provide great benefit to the agricultural community. The subject enzymes allow transgenic

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expression to deliver herbicide tolerance to herbicides that 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, 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.

[0073] 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 useage (hemicot). *Arabidopsis* and corn have been transformed with *AAD-1/PAT* constructs and have demonstrated 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 5, the exemplified rebuilt gene was more efficacious in conveying herbicide resistance to the plant, as compared to the bacterial gene.

[0074] The genes of the subject invention can be used to delivery a transgenic trait that imparts 2,4-D (or other aryloxyalkanoate auxinic herbicides) tolerance to plants. The results disclosed herein demonstrate the ability of AAD-1 to degrade representative AOPP herbicides, and specifically the herbicidally active R-enantiomer. Practicing aspects of the subject invention provides an opportunity to impart grass selectivity to the graminicidal AOPP herbicides. This concept is also unique in that resistance to multiple herbicide modes of action can be imparted by the same gene or by genes encoding enzymes with a similar mechanism.

[0075] 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.

[0076] 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 cultures, greenhouses, and in the field. In addition to HTC traits, methods for controlling weeds using

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herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, is within the scope of the invention. Likewise, combination of said HTC trait is useful when combined with other HTC traits (including but not limited to glyphosate tolerance) for the purpose of controlling weed (and like) species with newly acquired resistance or inherent tolerance to herbicides (*e.g.*, glyphosate).

[0077] 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.

[0078] 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.

[0079] The transgenic expression of the subject *AAD-1* genes are exemplified in *Arabidopsis* and corn (maize). However, the subject invention can be applied to any other desired types of plants, such as tobacco, cotton, canola, and soybeans. 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.

[0080] The promiscuity of AAD-1 for other phenoxyalkanoic auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes. In fact, there is high inherent value for the subject genes simply as a selectable marker for biotechnology projects.

[0081] Perhaps more importantly is the presently demonstrated activity on AOPP herbicides. This provides a tool for imparting grass selectivity to AOPP herbicides where there previously was none.

[0082] Still further, the subject invention provides a single gene that can provide resistance to broadleaf weed herbicides (auxins) and grass weed herbicides (AOPPs). This gene may be utilized

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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. Examples include oxyalkanoate herbicides with mode of action as inhibitors of protoporphyrinogen IX oxidase, or herbicides with other modes of action.

[0083] One gene of the subject invention, now coined *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: 112.5ug/ ml 2,4-D, 1mM Ascorbic acid, 1mM α-ketoglutarate, 50uM $Fe(NH_4)_2(SO_4)_2$), the recombinantly produced enzyme degrades 2,4-D into herbicidally inactive DCP (as determined by HPLC, mass spectrometry, colorimetric assay, and *Arabidopsis* plate assay). Additionally, AAD-1 has been demonstrated to convert the following herbicides into their corresponding inactive phenol: dichlorprop, mecoprop, haloxyfop, dichlofop, and others (See **Tables 3 and 4**).

[0084] 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 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, or, because of the subject invention, the level and/or concentration can be higher than that which was previously used. Preferably, plant cells and plants of the subject invention are protected against growth inhibition or

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injury caused by herbicide treatment. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

[0085] Transfer of the functional activity to plant or bacterial systems typically requires nucleic acid sequences, encoding the amino acid sequences for the proteins, 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.

[0086] 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.

[0087] 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.

[0088] 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,

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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.

[0089] 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.

[0090] 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.

[0091] 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.

[0092] 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.

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[0093] 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.

[0094] 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.

[0095] 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

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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.

[0096] 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 proteins of the subject invention.

[0097] 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.).

[0098] 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

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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.

[0099] 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.

[00100] 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.

[00101] As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with ^{32}P-labeled gene-specific probes can be performed by standard methods (*see, e.g.*, Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25°C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz et al. 1983:

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

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[00102] Washes can typically be carried out as follows:

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

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

[00103] For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C below the melting temperature (Tm) of the hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ ml denatured DNA. Tm for oligonucleotide probes can be determined by the following formula:

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

(Suggs *et al.*, 1981).

[00104] 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).

[00105] 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.

[00106] 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.

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[00107] 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.

[00108] 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.

[00109] 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.

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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.

[00110] 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.

[00111] "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.

[00112] 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,

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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, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500 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.

[00113] Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-

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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.

[00114] 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.

[00115] 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.

[00116] 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 polynucleotides and proteins of the subject invention can also be defined in terms of more particular identity and/or similarity

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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.

[00117] 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.

[00118] 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

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[00119] In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the functional/biological activity of the protein.

[00120] 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."

[00121] 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."

[00122] 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.

[00123] 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, and the like.

[00124] 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,

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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.

[00125] 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.

[00126] 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.

[00127] 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 United States Patent No. 5,135,867.

[00128] 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

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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); An *et al.* (1985).

[00129] 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, 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.

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[00130] 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.

[00131] 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.

[00132] 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*. 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.

[00133] 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. Pat. 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. Pat. No. 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. Pat. 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. Pat. No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Pat. No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Pat. Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Pat. Nos. 5,004,863 and 5,159,135, both to Agracetus. Other

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transformation technology includes whiskers technology. *See* U.S. Pat. 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. Pat. 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. Pat. Nos. 5,569,597 to Mycogen Plant Science and Ciba-Giegy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

[00134] 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.

[00135] 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

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employed is not essential to this invention, with the preferred marker depending on the host and construction used.

[00136] 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.

[00137] 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.

[00138] As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialaphos or glufosinate); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

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[00139] In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising *et al.* , 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the *uidA* locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the *uidA* locus of *E. coli* as described by Jefferson *et al.*, (1987) to identify transformed cells.

[00140] 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. Pat. 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,

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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.

[00141] Promoter regulatory elements may also be active 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 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.

[00142] 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. Pat. Nos. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

[00143] 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.

[00144] 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.

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Example 1 - PCR and cloning of gene into pET

[00145] 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.

[00146] 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.

[00147] 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)].

[00148] 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.

[00149] 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 included protocol. Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. # 608120) using M13

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Forward (5' GTA AAA CGA CGG CCA GT 3') and Reverse (5' CAG GAA ACA GCT ATG AC 3') primers, per manufacturers instructions. This gene sequence (SEQ ID NO:3), and its corresponding protein was given a new general designation for internal consistency *AAD-1 (v1)* (AryloxyAlkanoate Dioxygenase).

[00150] 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.

[00151] 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

2.1 – HPLC analysis.

[00152] 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.

[00153] 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 1mM 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

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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).

[00154] 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.

[00155] 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.

[00156] The extracts were then tested on HPLC for loss of the 2,4-D peak or presence of any additional peaks resulting from the degradation or modification of 2,4-D. Conditions for the HPLC were: Luna 10µ C18(2) 250 x 4.6mm (Phenomenex cat.# 00G-4253-E0), run at 50% ACN + 0.05% TFA: 50% H_2O + 0.05% TFA to 100% ACN + 0.05% TFA over 5 min.

[00157] To evaluate AAD-1 (v1) for degradation of any other herbicide for utilization as a substrate, the same conditions as above were used except that the 2,4-D in the assay was substituted with an appropriate amount of the other herbicide. Concentrations of these herbicides in the assay were dependent on its relative activity in the plate bioassay. Most were tested at 500 µg/ml.

2.2 – Plate test bioassays for herbicide degradation.

[00158] 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.

[00159] *Arabidopsis* seeds were surface sterilized for 10 min. in 50% commercial bleach/deionized water (v/v) with 1 µL of Tween-20 added as a wetting agent with vigorous agitation (shaker table @

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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.

[00160] Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4 \cdot 2H_2O$, 10 μM NaCl, 10 nM $CoCl_2 \cdot H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60x15-mm Petri dishes (Falcon 1007). PTM was additionally modified by adding up to six rates of test herbicide standards or herbicide-enzyme test solution dilutions such that the four-fold concentration increments covered a rate range of three orders of magnitude with the GR_{50} rate (50% growth reduction) approximately in the center of the range.

[00161] For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed and maintained under sterile conditions in a low light growth chamber (24 h day^{-1}, 100 $\mu E/m^2s^1$, 23°C) for 7 days. Root length or root+shoot length were measured for five randomly chosen *Arabidopsis* and bentgrass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

[00162] 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.

2.3 – HPLC results.

[00163] From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see **Figure 1**) and ferrous ion to bind in the active site. Other experiments in the literature have shown that the addition of ascorbate increased the enzymatic activity by maintaining the iron in the reduced state, thus preventing the enzyme for being degraded. Based on this previous work, initial assays were set up under the assumption that the subject enzyme would work in the same way as other members of this general class of enzyme.

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[00164] 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.

[00165] 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 flourine 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).

[00166] 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.

2.4 – Plate test bioassays for herbicide degradation.

[00167] Bioassay tests results corroborated initial HPLC results that indicated loss of 2,4-D parent following incubation of 2,4-D solutions with unpurifiedrecombinant AAD-1 (v1) extracts (**Figure 2**). Additionally, the herbicidal activity of the phenoxypropionic acid, dichlorprop, was also effectively degraded. The ratio of the nominal GR_{50} for herbicide+enzyme solution versus the herbicide solution alone served as measure of loss of parent herbicide activity resulting from enzyme activity. A ratio

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of 2-3 typically correlated with 50-75% loss of parent herbicide activity (**Table 6**). Often a GR_{50} could not be determined following enzyme treatment; *de facto*, no detectable herbicide activity remained.

[00168] 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 & 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 – Testing with colorimetric assay

3.1 – AAD-1 (v1) Assay.

[00169] 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) to enable deployment in a 96-well microplate format. The subject invention includes the use of *AAD-1* in detection assays such as these. 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 minute, 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.

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3.2 – Phenol Detection.

[00170] The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the phenol product, 2,4-dichlorophenol. However, other phenols could potentially be released from different aryloxyalkanoate herbicides (**Figure 4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM NH_4FeSO_4, 200 μM sodium ascorbate. The absorbance is shown in **Table 8**.

[00171] The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so are readily detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. Thiophenols were also not detected. Some phenols equivalent to those found in certain protoporphyrinogen IX oxidase inhibitors produced faint pink colors of significantly less intensity than the 2,4-dichlorophenol. Some phenols produced entirely different colors with differing λmax values (2-Cl,4-F, 5-nitrophenol, yellow, 420 nm; 6-hydroxy-2,4-D, blue, 580 nm; 3-amino-2,4-dichlorophenol, orange, 480 nm).

[00172] Further experiments showed that 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.

[00173]

3.3 – Extraction from *Escherichia coli*.

[00174] *E. coli* cell pellets were resuspended in 0.1M 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 1mM. 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.

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3.4 – Results – Activity of recombinant AAD-1 (v1) expressed in *Escherichia coli*.

[00175] 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.

[00176] 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 AOPPs as well as phenoxy auxins.

[00177] 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** as well. 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)

4.1--Expanded substrates

[00178] 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.

[00179] **Table 3** shows the A_{510} detected after the action of AAD-1 (v1) on a variety of aryloxyalkanoate auxinic herbicides and auxin analogs. The best substrate tested was dichlorprop, with mecoprop also being efficiently cleaved. Two other phenoxypropionates, the 4-fluoro and 3-amino analogs of dichlorprop, were also acted on effectively by AAD-1 (v1). AAD-1 (v1) produced

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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).

[00180] From these data, 2,4-D is cleaved by AAD-1 (v1), as are two phenoxyalkylsulfonates (X188476 and X398166, sesone). **Table 4** shows similar data for a variety of AOPP graminicide herbicides, and also the safener cloquintocet (albeit to a lesser extent). All the commercial AOPP herbicides tested were 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.

[00181] AAD-1 (v1) activity on quizalofop was the greatest (76% of the dichlorprop rate) and cyhalofop the least (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Very little, if any, activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, little activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

4.2—Catalytic characterization

[00182] The K_m and k_{cat} values of purified AAD-1 (v1) were determined for four herbicide substrates, (R)-dichlorprop, (R)-haloxyfop, (R)-quizalofop and 2,4-D under standard assay conditions (25 mM MOPS, pH 6.8; 200 μM Na ascorbate; 200 μM Fe2+; 1 mM α-ketoglutarate; 25° C). The dose response curves were fitted using Grafit (Erithacus Software, UK), and the graphs and derived constants are shown in **Figure 7** and **Table 9** respectively. The K_m values for the four substrates were fairly similar (75 – 125 μM), but the k_{cat} values varied significantly. (R)-dichlorprop had the highest k_{cat} value and 2,4-D the lowest (10% that of (R)-dichlorprop). These k_{cat} values were consistent with the range of values seen in the substrate specificity tests shown in Tables 4 and 5, as these were performed at high (saturating) substrate concentrations (1 mM).

[00183] 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). The

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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 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.

4.3 – Additional substrates for AAD1.

[00184] Three additional substrates were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay; X11115427, X124987 and MCPA. The results are shown in **Table 10**. 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 >700 fold better than 2,4-D as a substrate (**Table 8**). 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

5.1 – Background.

[00185] 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.

[00186] 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

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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.

[00187] **Table 11** illustrates how high the G+C content is in maize. For the data in **Table 11**, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVectorTM program (Accelerys, San Diego, California). Intron sequences were ignored in the calculations.

[00188] 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.

[00189] 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 12**. 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,

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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.

[00190] 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.

[00191] 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.

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5.2 – Rebuild analysis.

[00192] 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 essentially the identical protein (disclosed herein as SEQ ID NO:11) as the native gene (disclosed herein as 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 13** shows the differences in codon compositions the native and plant-optimized sequences.

5.3 – Completion of binary vectors.

[00193] 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 and M13 Reverse 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

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the correct orientation. The verified completed construct (pDAB721) was used for transformation into *Agrobacterium* (see section 6.2).

[00194] 5.3.2 – Native *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).

[00195] The PCR amplicon was ligated 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.

[00196] 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" were used.

[00197] 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).

[00198] A sequencing reaction using the M13 Reverse primer 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.

[00199] 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

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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*.

Example 6 – Transformation into *Arabidopsis* and Selection

6.1 – *Arabidopsis thaliana* Growth Conditions

[00200] 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).

[00201] Sunshine Mix LP5 (Sun Gro Horticulture Inc., 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.

[00202] Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 μmol/m^2sec under constant temperature (22° C) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2 – *Agrobacterium* transformation.

[00203] An LB + agar plate with erythromycin (Sigma Chemical Co., St. Louis, MO) (200mg/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.

[00204] 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

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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.

[00205] 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) and streptomycin (Sigma Chemical Co., St. Louis, MO) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

[00206] Colonies were selected and streaked onto fresh YEP + agar with erythromycin (200 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) 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 10µl 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.

6.3 – *Arabidopsis* transformation.

[00207] 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) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28°C with constant agitation at

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220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 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.

6.4 – Selection of transformed plants.

[00208] Freshly harvested T_1 seed [transformed with native [*AAD-1 (v2)*] or plant optimized [*AAD-1 (v3)*] gene] was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5 x 51-cm germination trays (T.O. Plastics Inc., Clearwater, MN), each receiving a 200 mg aliquots of stratified T_1 seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4°C for 2 days to complete dormancy requirements and ensure synchronous seed germination.

[00209] 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).

[00210] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml/tray (703L/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

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individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5°C, 50±30% RH, 14 h light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)* (plant optimized gene) or *AAD-1 (v2)* (native microbial gene) to provide phenoxy auxin herbicide resistance.

[00211] Random individual T_1 plants selected for glufosinate resistance above were confirmed for expression of the PAT protein using a PAT ELISA kit (Part no. 7000045, Strategic Diagnostics, Inc., Newark, DE) to non-destructively confirm fidelity of selection process (manufacturer's protocol). Plants were then randomly assigned to various rates of phenoxy herbicides (dichlorprop or 2,4-D). Phenoxy rates initially applied were 12.5 g ae/ha 2,4-D and 50 or 200 g ae/ha dichlorprop. GR_{99} for *Arabidopsis* is about 50 g ae/ha 2,4-D and 200 g ae/ha dichlorprop. Elevated rates were applied in subsequent trials (50, 200, 800, or 3200g ae/ha).

[00212] All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703L/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.

[00213] Some T_1 individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether haloxyfop could be effectively degraded *in planta*.

[00214] Although *Arabidopsis*, being a dicot, is not an optimal system for testing ACCase-inhibiting AOPP grass herbicides, *AAD-1 (v3)*-transformed T_1 plants were subjected to elevated rates (400-1600 g ae/ha) of RS-haloxyfop acid (internally synthesized) that do cause growth abnormalities and

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death of wildtype *Arabidopsis* using the DeVilbiss sprayer as described above Injury ratings were taken 7 and 14 days after treatment. Likewise, T_1 individuals were treated with the pyridinyloxyacetate auxin herbicide, fluroxypyr.

6.5 – Results of selection of transformed plants.

[00215] The first Arabidopsis transformations were conducted using *AAD-1 (v3)* (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (*see* **Table 14** below in Example 15). A small lot of *AAD-1 (v2)*(native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

[00216] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 15** compares the response of native *AAD-1 (v2)* and plant optimized *AAD-1 (v3)* genes to impart 2,4-D resistance to Arabidopsis T_1 transformants. Both genes imparted resistance to individual T_1 Arabidopsis plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 15** simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)* or *AAD-1 (v3)* versus the wildtype or *PAT/Cry1F* transformed controls. Also evident is that tolerance appears to be significantly greater (frequency and overall level of individual response) for the plant optimized sequence *AAD-1 (v3)* versus the native sequence *AAD-1 (v2)* (see **Table 15**). Higher rates of 2,4-D (up to 3,200 g ae/ha) have been applied to additional T_1 individuals expressing AAD-1 (v3). Injury levels tend to be greater and the frequency of highly resistant plants is lower at these elevated rates (6x field rates). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these

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elevated rates. Nonetheless, some individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

[00217] **Table 16** shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400 – 1,600 g ae/ha (**Table 17**). 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 pryridinyloxyacetate auxin) in transformed *Arabidopsis*, consistent with *in vitro* work with heterologously expressed enzyme.

6.6 – *AAD-1 (v3)* as a selectable marker.

[00218] The ability to use *AAD-1 (v3)* as a selectable marker using 2,4-D as the selection agent was analyzed initially with Arabidopsis transformed with as described above. T_1 seed transformed with *PAT* and *AAD1 (v3)* (pDAB 721) were sown into flats and germinated as described above and compared to similar seed treated with the normal glufosinate selection scheme (5 and 10 DAP). 2,4-D (50g ae/ha) was applied to seedling *Arabidopsis* as previously done with glufosinate. Variation in number of applications and timing of application were tested. Each tray of plants received either one or two application timings of 2,4-D in one of the following treatment schemes: 5+10 DAP, 5+14 DAP, 10 DAP, 10+14 DAP, 14 DAP. Plants were identified as Resistant or Sensitive 19 DAP and ELISA test strips run to determine frequency of successfully co-transforming an active *PAT* gene.

[00219] 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 14).** 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

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would indicate both selection agents are equally good for selecting Arabidopsis transformed with *AAD-1 (v3)* or *PAT* genes, respectively. Two successive applications most accurately identify resistant individuals with both herbicides tested.

6.7 – Heritability.

[00220] A variety of T1 events were self-pollinated to produce T_2 seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T_2 siblings. Each individual T_2 plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). More than 60% of the T_1 families (T_2 plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ($P > 0.05$).

[00221] Seed were collected from 12 to 20 T_2 individuals (T_3 seed). Twenty-five T_3 siblings from each of eight randomly-selected T_2 families were progeny tested as previously described. Approximately one-third of the T_2 families anticipated to be homozygous (non-segregating populations) have been identified in each line tested: ranging in frequency from one to four out of the eight families tested. These data show *AAD1 (v3)* is stably integrated and inherited in a Mendelian fashion to at least three generations.

Example 7 – Transformation into Maize

7.1 – Cloning of *AAD-1 (v3)*.

[00222] The *AAD-1 (v3)* fragment was received on an NcoI/SacI fragment. Construct pDAB4005 was digested with NcoI and SacI and the 5175bp backbone fragment isolated. The two fragments were ligated together using T4 DNA ligase and transformed into DH5α cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. The correct intermediate plasmid was named pDAB3403. Both pDAB3403 and pDAB8505 were digested with NotI. The 3442bp band from pDAB3403 and the 11017bp band from pDAB8505 were isolated and purified. The fragments were ligated together, transformed into DH5α, and the resulting plasmids were screened for orientation. The final construct was designated pDAB3404.

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7.2 – Callus/suspension initiation.

[00223] To obtain immature embryos for callus culture initiation, F_1 crosses between greenhouse-grown Hi-II parents A and B (Armstrong *et al.* 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersion in 70% ethanol for 2-3 minutes, followed by immersion in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

[00224] Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Complete Medium (Phyto Technology Laboratories C167), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L $AgNO_3$, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter *et al.*, 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Complete Medium (Phyto C167), 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.

[00225] 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. Phyto* M524), Modified MS Vitamins, 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 using a 50 ml pipette. Once the suspensions were fully established, they were cryopreserved for future use.

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7.3 – Cryopreservation and thawing of suspensions.

[00226] One day 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 the vials were placed in a controlled rate freezer set 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.

[00227] 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. 1 or 4) in 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 solid medium (N6 Complete Medium (Chu *et al.*, 1975. Phyto C167), 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite (omitted for liquid medium), pH 5.8. The tissue paper containing the cells was transferred to fresh medium after one week. After approximately 2 weeks, the tissue was re-initiated into 125 ml flasks. Three milliliters PCV was subcultured every 3.5 days until a total of 12 ml PCV was obtained. Then subculturing was conducted as described previously.

7.4 – WHISKERS-mediated transformation

[00228] 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 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 Complete Medium (Phyto C167), 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

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GN6 S/M liquid medium to ~405 mg of pre-autoclaved, silicon carbide whiskers (Advanced Composite Materials, Inc.).

[00229] After incubation in GN6 S/M, the contents of each flask was 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 8.1ml 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.

[00230] Depending on the parameter being tested, 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 solid medium. Plates were cultured for 1 week at 28°C in a dark box covered with a single layer of plastic.

7.5 – Selection of transformed tissue.

[00231] After 1 week, the filter papers were transferred to 60x20 mm plates of GN6 solid medium containing 2,4-D and/or dicamba plus 1 mg/L bialaphos [in the form of Herbiace, GN6 1H (N6 Complete Medium (Phyto C167), 2.0 mg/L 2,4-D, 30 g/L Sucrose, 100 mg/L myo-inositol, 1.0 mg/L (active ingredient) Herbiace, 2.5 g/L Gelrite, pH 5.8) or GN6D 1H (N6 Complete Medium (Phyto C167), 8.0 mg/L dicamba, 0.8 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 1.0 mg/L (active ingredient) Herbiace, 2.5 g/L Gelrite, pH 5.8)] or 100 nM haloxyfop-R methyl ester (in the form of Gallant* Super, same as GN6 1H and GN6D 1H except active ingredient is 100nM Gallant).

[00232] 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 plate or else the entire plate into 3.0 of melted GN6 Agarose medium (N6 Complete Medium (Phyto C167), 2.0 mg/L 2,4-D, 30 g/L Sucrose, 100 mg/L myo-inositol, 7 g/L SeaPlaque Agarose, pH 5.8, Autoclaved for only 10 minutes at 121° C) containing the same concentration of selection agent as in the previous transfer. The

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tissue was broken up and evenly poured onto the surface of a 100 x 15 mm plate of GN6 medium containing the same level of selection agent as was added to the agarose. This was repeated for all remaining tissue. Once embedded, plates were individually sealed, and incubated in the dark at 28° C.

[00233] Transgenic isolates are typically recovered 5-7 weeks post-embedding. Any putative 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, an event was deemed to be resistant and was submitted for molecular analysis.

7.6 – Dose response of non-transformed tissue to haloxyfop

[00234] Three established Hi-II cell lines were pooled together, WHISKERS-treated without DNA, filtered onto GN6 solid medium at the rate of 6 ml per filter, and allowed to callus for 2-3 weeks at 28° C. Approximately 450 mg of callused suspension tissue was transferred per treatment to selection media in 100 x 15 mm plates containing the following concentrations of haloxyfop-R methyl ester from Gallant™* RLPU: 0, 10, 50, 100, 200, and 400 nM. Three replicates were used per concentration. A control medium containing 1 mg/L bialaphos, 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.

7.7 – Results.

[00235] 7.7.1 – Dose response to haloxyfop-R methyl ester. A dose response was implemented in order to determine the level of selection needed for WHISKERS-treated suspension tissue. Results from an initial dose response testing levels from 0 to 400 nM haloxyfop-R are shown in **Figure 8**.

[00236] These data show that growth appears to hit a plateau at levels of 100 nM and higher, and suggests that this level might be a good starting point for direct recovery of transformed isolates from selection. Although a plateau is evident, growth reduction at 100 nM haloxyfop was still only 87% of the control whereas the Herbiace treatment showed 98% growth reduction as compared to the control.

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[00237] 7.7.2 – Transformant selection strategy using pDAB3404. Two selection strategies were used for WHISKERS-mediated transformation (Frame *et al.*, 1994) of embryogenic suspensions. For Strategy I, only Herbiace selection was implemented; however, 2,4-D and dicamba were compared as auxins. [2,4-D is a critical component of maize tissue culture media because it keeps the callus tissue from differentiating prematurely. Since 2,4-D is degraded by AAD-1 (V3) and dicamba is not, dicamba (at 8 mg/L dicamba + 0.8 mg/L 2,4-D) was assessed for its ability to served as a growth regulator in 2,4-D maintained cells.] Strategy II implemented 75% Herbiace selection and 25% haloxyfop selection while comparing 2,4-D and dicamba within each selection agent. A total of 3 bottles were WHISKERS-treated for each strategy for a total of 6 bottles. The tissue from Strategy I was embedded whereby each filtered plate produced 2 embedded plates. However, the tissue from Strategy II was embedded whereby each filtered plate produced only 1 embedded plate. Therefore, a total of 360 embedded plates were produced from each strategy. Herbiace-resistant events were recovered approximately 3 to 6 weeks post-embedding. They appeared as light yellow sectors proliferating against a background of darker yellow-to-brown growth-inhibited tissue. The putatively transformed events were subcultured onto fresh GN6 (1H) medium in 60x20 mm plates every 10-14 days using a dissecting scope. They became established and ready for analysis after approximately 2-6 weeks.

[00238] Ninety-six isolates were recovered from Herbiace selection and 3 isolates were recovered from direct selection on haloxyfop-R. All were submitted for copy number analysis for the PAT gene. In addition, full length plant transformation unit (PTU) PCR analysis was performed [for the *AAD-1 (v3)* gene] concurrently using primers that amplified 1520 bp of the Ubi promoter, all of the *AAD-1 (v3)* coding region and 25 bp of the rice actin promoter, with a final product of 2880 bp. Thirty-four isolates, all of which were selected upon Herbiace, were identified to contain 3 copies or less of the *PAT* gene plus an intact PTU for the *AAD-1 (v3)* gene. Most, but not all of these callus events were also submitted for Western/ELISA screening.

[00239] Fourteen Western positive events that contained 3 copies or less of *PAT* plus an intact PTU for *AAD-1 (v3)* were regenerated and advanced to the greenhouse. Two additional events that contained 3 copies or less of *PAT* plus an intact PTU for *AAD-1 (v3)* but were never analyzed for expression were also advanced. These latter 2 events were subsequently tested for expression using

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T_0 leaf tissue and were found to express AAD-1 (v3). A final characterized event that showed expression during the callus stage but not in leaf tissue was also advanced to the greenhouse.

[00240] Dose Response of Transformed Tissue to Haloxyfop. Approximately 150-200 mg of transgenic callus tissue was transferred to 60 x 20 mm plates containing the following concentrations of haloxyfop-R methyl ester from Gallant* Super: 0, 30, 100, 300 nM. Three replicates were used per concentration. An additional medium containing 1mg/L bialaphos (from a Herbiace formulation) was also included for comparison. These steps were repeated using non-transgenic maize tissue derived from callused embryogenic suspensions.

[00241] After 2 weeks, tissue from each plate was removed, weighed, and then transferred to fresh media of the same concentration for an additional 2 weeks. After a total of 4 weeks elapsed time, tissue was removed, weighed a final time, and then discarded. One example of results after 4 weeks is provided in **Figure 9**. 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 ~97% versus untreated control at this rate. The expression of AAD-1 (v3) enzyme allowed protection of the corn callus from haloxyfop.

[00242] Regeneration of Transformed Tissue. Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, "28", containing 1 mg/L bialaphos. Cells were allowed to grow in low light (13 $\mu Em^{-2}s^{-1}$) for one week then higher light (40 $\mu Em^{-2}s^{-1}$) for another week before being transferred to hormone-free regeneration medium, "36", containing the same level of selection. Small (3-5 cm) plantlets were removed and placed into 150 x 25 mm culture tubes containing SHGA (Schenk and Hilldebrandt salts: 10 g/L sucrose, 1 g/L myo-inositol, 2 g/L Gelrite, pH 5.8) medium. Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse and allowed to acclimate to greenhouse conditions under a 0.5 L clear plastic cup for 5-10 days. Once plants were growing rapidly, cups were removed and plants that typically possessed 4-10 axillary shoots were thinned to 1-2 primary shoots. In most cases 15 plants were produced for each event. A total of 251 plants representing 17 events were chosen for advancement to the next generation. Reciprocal crosses with a commercial-type hybrid shall be made to generate sufficient F_1 seed for field tolerance testing.

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[00243] Tolerance of T_0 corn plants to AOPP herbicides. If more than 15 clone plants per event were successfully regenerated, then extra plants were transferred to the greenhouse for preliminary tolerance screening with postemergence-applied AOPP herbicides on T_0 corn plants. Greenhouse-acclimated plants were allowed to grow until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C under 16 hour light:8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of one of three AOPP herbicides: Assure II, Clincher*, or Gallant Super* 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 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.

[00244] 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.

[00245] 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*.

[00246] **Figure 10** 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.

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[00247] **Table 18** shows data for the responses of selected *AAD-1 (v3)*-transformed T_0 corn events to three AOPP herbicides applied postemergence.

7.8 – *Agrobacterium*-mediated transformation of maize

7.8.1 Plant Material

[00248] Seed of two S_3 parents of "High II" (i.e., Parent A and B) or the F_1 cross (Armstrong et al., 1991) are planted directly into 5 gallon-pots containing commercial soil mix (Conrad Fafard, Inc., Springfield; Soil Mix #3). The plants are grown in the greenhouse with a 16 hour photoperiod supplemented by a combination of high pressure sodium and metal halide lamps. The illumination is approximately 1,500 ft-candles. The day and night temperature is maintained at 27/20 + 2° C. The plants are watered as needed and a soluble fertilizer (20-20-20) along with chelated iron (8 mg/L) is applied at regular intervals.

7.8.2 Tissue Source

[00249] For obtaining immature embryos, controlled pollinations are performed. Plants at anthesis are prepared for pollination by cutting back silks one day prior to pollination, thereby producing a full brush of silks for maximum fertilization and seed set. On the day of pollination, actively shedding tassels are bagged, and fresh pollen is collected and applied carefully onto the silks. When the embryos reach 1.0-1.2 mm size (9-10 days after pollination), the ear is excised and surface sterilized. Briefly, ears are washed with diluted "Liquinox" soap and subjected to immersions in 70% ethanol for 2-5 min. and 20% commercial bleach (0.1% sodium hypochlorite) for 30-45 minutes followed by 3 rinses in sterile, distilled water. Following sterilization, immature embryos (1.0-1.2 mm) are isolated.

7.8.3 Bacterial Supply

[00250] 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 bacteria from a YP streak plate is put into 5ml of LS-inf. Mod medium (L&S Basal Medium, 1 ml/LN6 Complete Medium (Phyto C1149), 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 is vortexed until a uniform suspension is achieved. The bacterial concentration was taken using a Klett-Summerson

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Photoelectric Colorimeter by reading the density of the solution. The solution is adjusted to a concentration of Klett 200 (~ $1x10^9$ cfu/ml) with 100μM actetosyringone added to the solution.

7.8.4 Infection and Cocultivation

[00251] The immature embryos are isolated directly into a microfuge tube containing 2 ml LS-inf. Mod, which is a high osmotic 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 5-10 minute incubation at room temperature, the embryos are transferred to LS-As Mod medium (L&S Basal Medium, 1 ml/LN6 Complete Medium (Phyto C1149), 1.5 mg/L 2,4-D, 30.0 g/L sucrose, 6 mM L-proline, 0.85 mg/L $AgNO_3$,1, 100μM actetosyringone, 3.0g/L Gelrite, pH 5.8) for the co-cultivation step which lasts for 5-7 days at 25° C.

7.8.5 Selection

[00252] After co-cultivation, the embryos are moved through a 3-step selection scheme after which transformed isolates will be obtained. For selection, LSD Mod medium (L&S Basal Medium, 1 ml/LN6 Complete Medium (Phyto C1149), 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, 100-600 nM Pursuit®, 2.5 g/L Gelrite, pH 5.7) is used along with one of three Pursuit® selection levels as well as cefotaxime to stop further growth of the *Agrobacterium*. Throughout the selection phase, the embryos are cultured in the dark at 27° C. Pursuit® is used as the chemical selection agent when the *AHAS* gene is used, based on U.S. Patent No. 5,731,18. The embryos are first put on a very "low" level of selection (~ 50nM Pursuit®) for 5-7 days, moved up to a "moderate" selection level of 300 nM Pursuit® for 2 weeks and then taken through 2 week cycles of the "high" selection level of 600 nM Pursuit® until embryogenic colonies are obtained. These colonies are maintained on the high selection pressure for the remainder of the culture period. The recovered transgenic colonies are bulked up by transferring to fresh selection medium at 2 week-intervals for regeneration and further analysis.

7.8.6 Regeneration and Seed Production

[00253] For regeneration, the cultures are transferred to 28 "induction" medium (MS Basal salts, 0.25 mg/L 2,4-D, 5 mg/L BAP, 30.0 g/L sucrose, 250 mg/L cephotaxime, 100-600 nM Pursuit®, 2.5 g/L

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Gelrite, pH 5.7) and incubated at 28° C in low light (125 ft-candles) for one week followed by one week in high light (325 ft-candles). The callus cultures are then non-selectively transferred to the 36 "regeneration" medium (MS Basal salts, 30.0 g/L sucrose, 250 mg/L cephotaxime, 50-600 nM Pursuit®, 2.5 g/L Gelrite, pH 5.7) for differentiation of plantlets. The composition of the 'regeneration' medium is similar to the "induction" medium except that it did not contain any plant growth regulators. Both "induction" and "regeneration" media contain Pursuit® selection along with continued use of cefotaxime. When plantlets are established, they are transferred to SHGA tubes to allow for further growth and development of the shoot and roots. After a healthy shoot has been obtained, it is moved to the greenhouse where it is first grown in 10 cm pots containing commercial soil mix (Fafard #3). At the 3-5 leaf stage, they are transplanted to larger pots (5 gallon) and grown to maturity in the greenhouse. Controlled pollinations for seed production and tolerance testing can be conducted as described above in Example 7.2.

Example 8 – Purification of AAD-1 (v1) for Antibody Creation and Biochemical Characterization

[00254] 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, 1mM 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.

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Example 9 – Western Blot and ELISA Analysis for AAD-1 Expressed in *Arabidopsis* and Maize

9.1 – Antibody production.

[00255] Five milligrams purified AAD-1 (v1) delivered to Zymed Laboratories, Inc. (South San Francisco, CA) for polyclonal antibody production. Two rabbits each received 5 injections in the period of 10 weeks with each injection contained 0.5 mg of the purified AAD-1 (v1) protein resuspended in 1 ml of Incomplete Freund's Adjuvant. Sera after injections were shipped back for analysis.

9.2 – Antibody purification and biotinylation.

[00256] Rabbit anti-serum was purified using ImmunoPure® (G) IgG Purification Kit (Pierce Biotech). One ml of the serum was diluted to 5 ml with the Binding buffer and the purification was conducted according to the manufacturer's instructions. The purified fractions were pooled and dialyzed against PBS using dialyzing cassette (Pierce Biotech.) with MWCO of 100,000 for overnight at 4° C. One milligram of the purified antibody was biotinylated using EZ-LinkTM Sulfo-NHS-LC-Biotin labeling kit (Pierce Biotech.) following the manufacturer's instruction. The reaction proceeded on ice for 2 hours and the product was dialyzed against PBS as above.

9.3 – Extraction of AAD-1 v3 from plant materials.

[00257] Transgenic *Arabidopsis* leaves or corn calli were collected from different transgenic and 2 control lines. Approximately 30 to 40 mg of plant material was weighed and put into a cluster tube containing one steel bead. A tungsten bead was put on top of the plant material and extraction buffer (PBS containing 0.1% Triton X-100 and 10 mM DTT) was added (10 µl per mg of plant material). All the samples were put into cluster box with the lids secured. The box was put into shaker to pulverize the plant tissue for 4 minutes at maximum speed. Tubes were then centrifuge for 10 min at 2500 rpm. The supernatant was used directly for Western Blot or ELISA analysis.

9.4 – Western blots.

[00258] All steps were conducted at room temperature except as otherwise stated. Various concentrations of AAD-1 (v1), namely 1.0, 0.1, 0.01 and 0.001 ng, were denatured in sample buffer (Bio-Rad; Hercules, CA) and loaded onto pre-casted Novex® Tris-Glycine polyacrylamide gel (Invitrogen; Carsberg, CA). Ten µl SeeBlue® Plus Pre-stained Standard (Invitrogen; Carsberg, CA) was loaded in separate well to monitor the electrophoresis, which was conducted at 200 V. The

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proteins were then electro-transferred onto nitrocellulose paper. The blots were blocked with 4% skim milk dissolved in phosphate buffered saline (MPBS) for 1 hourand probed with rabbit anti-sera diluted 5000-fold in PBS. After 1 hour incubation, the blots were washed 3 times with PBS containing 0.05% Tween 20 (PBST). Bound antibodies were detected by goat anti-rabbit antibodies conjugated to horseradish peroxidase (GAR/HRP; Pierce; Rockford, IL). The blot was incubated for 1 hour and followed by 3 washes. ECL Western Blotting Detection Reagents (Amersham; Piscataway, NJ) were used to visualize the protein detected. Manufacturer's instructions were followed. To detect the AAD-1 (v3) in transgenic plant extracts, 20 µl of plant extract (see below) was added to 10 µl of 3X sample buffer. After incubated at 95° C for 5 minutes, 20 µl of the mixture was loaded onto gel for electrophoresis.

9.5 – Sandwich ELISA.

[00259] All steps were conducted at room temperature except otherwise stated. Purified rabbit antibody was immobilized on microtiter wells to capture AAD-1 (v3) in plant extract. The purified antibody was diluted to 1 µg/ml in PBS and 100 µl was incubated in each well at 4° C for 16 hr. After 4 washes, each well was blocked with 200 µl MPBS for 1 hour. Various concentrations of plant extracts were incubated in the wells. A 2-fold serial dilution of the extracts was performed on the plate, with the highest concentrations at the top of the plate. To prepare a standard curve, different concentrations of purified AAD-1 (v3) (100, 50, 25, 12.5, 6.25, 3.125, 1.0625 and 0 ng/ml) were also put into the wells in duplicate. The plate was incubated for 1 hour and washed 4 times with PBST. Biotinylated antibody was used to detect the bound AAD-1 (v3). Diluted to 1 µg/ml, 100 µl of the biotinylated antibody was incubated in each well for 1 hour. After another wash, neutravidin conjugated to horseradish peroxidase (Pierce Biotech.) was added to each well and incubated for another hour. TMB (3,3',5,5'Tetramethylbenzidine) substrate (100 µl) was added and, after the 15 min incubation, 100 µl 0.4 N sulfuric acid was added to stop the reaction. The absorbance at 450 nm was measured using a microtiter plate reader. The concentration of AAD-1 (v3) in plant extract was extrapolated from the standard curve on the same plate.

9.6 – Results of Western and ELISA data.

[00260] Polyclonal antibodies specific to AAD-1 (v1) were generated by immunizing rabbits with recombinant AAD-1 (v1) purified from *E. coli*. After 5 separate boosts, the sera from both rabbits

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were able to detect 1 ng of AAD-1 (v3) in Western Blot and direct ELISA analysis. Serum from second rabbit was further purified by Protein G affinity chromatography and an aliquot of it was biotinylated. A sandwich ELISA was developed, which can quantify AAD-1 (v3) in the linear range of 5 to 100 ng/ml.

[00261] Eight and 20 different transgenic *Arabidopsis* leaves and corn calli, respectively, were analyzed by both Western Blot and the Sandwich ELISA. Results indicated that the expression level of AAD-1 (v3) in *Arabidopsis* ranged from 2 to 50 µg/ml of the plant extract, which is equivalent to 0.08 to 2.0% of total soluble protein extracted from the leaf tissue. Similarly, AAD-1 (v3) was found to be 0.7 to 2.1% of total soluble protein in corn calli tested.

Example 10 – Tobacco Transformation Protocol

[00262] Tobacco transformation with *Agrobacterium tumefaciens* can be 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) can be 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 can be 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 will be sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB721), grown overnight in a flask on a shaker set at 250 rpm at 28° C, should be 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 should be 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 are to be 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 should be 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

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plants arising from transformed foci will be 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 will be grown in a lighted incubator room. After 3 weeks, stem cuttings will be taken and re-rooted in the same media. Plants should be ready to send out to the greenhouse in 2-3 additional weeks.

[00263] Plants can be 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 one week the bag should be opened; the plants were fertilized and allowed to grow in the open bag until the plants are greenhouse-acclimated, at which time the bag can be removed. Plants are grown under ordinary warm greenhouse conditions (30° C, 16 hour day, 8 hour night, minimum natural + supplemental light = 500 $\mu E/m^2s^1$). T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1* and *PAT* genes in transgenic tobacco. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 50-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for *Arabidopsis* and corn.

Example 11 – Soybean Transformation

[00264] Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgette *et al.*, 1995), amino acid modification (Falco *et al.*, 1995), and insect resistance (Parrott *et al.*, 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe *et al.*, 1988) or somatic embryogenic cultures (Finer and McMullen,

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1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchee *et al.*, 1988) or zygotic embryos (Chee *et al.*, 1989) have been reported.

[00265] 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* 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*.

[00266] 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 axillary 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

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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 it is optimized for routine high frequency transformation of several soybean genotypes (Zhang *et al.*, 1999; Zeng *et al.*, 2004).

[00267] *Agrobacterium* Preparation. The plasmid, pDAB721, contains the *AAD-1* 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.

[00268] *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.

[00269] 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 a 18/6 hour light/dark regime. B5 medium consists of macro and micro nutrients and vitamins described by Gamborg *et al.* (1968) (Sigma, Cat. #G 5893, St. Louis). All media are solidified with 0.8% (w/v) washed agar (Sigma Cat #A 8678). Alternatively certain genotypes of soybean can undergo a dry surface sterilization procedure using chlorine gas (Cl_2) whereby mature seeds are placed into 100 x 25 mm Petri plates in a single layer using about 130 seeds per plate. Approximately 3-4 plates are placed into a desiccator within a fume hood in such a way that all the plates are half open and that there is enough space for a 250 ml beaker in the middle of the desiccator. The beaker is filled with 95ml of commercial bleach, to which 5 ml of concentrated (12N) HCl is added drop wise along the side of

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the beaker. The desiccator is immediately closed and allowed to stand for at least 16 hours in a fume hood. Following sterilization, the plates are closed, then brought to a laminar flow hood and left open for about 30 minutes to remove any excessive Cl_2 gas. Seeds are then germinated as described previously. The dry surface sterilized seeds will remain sterile at room temperature for about 2 weeks. Explants are prepared for inoculation as previously described (Hinchee *et al.*, 1988).

[00270] 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.

[00271] 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.

[00272] Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma Cat #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-napthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

[00273] 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.

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[00274] Progeny Evaluation. T_0 plants will be allowed to self fertilize in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1* and *PAT* genes in transgenic soybean. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 100-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in Example 9 for Arabidopsis and corn. Determination of the inheritance of AAD-1 will be made using T_1 and T_2 progeny segregation with respect to herbicide tolerance.

Example 12 – Cotton Transformation Protocol

[00275] Seeds are surface-sterilized in 95% alcohol for 1 minute, rinsed, sterilized with 50% commercial bleach for 20 minutes, then rinsed 3 times with sterile distilled water before being germinated on G-medium (Murashige and Skoog (MS) basal salts, B5 vitamins, 3% sucrose) in Magenta GA-7 vessels and maintained under a high light intensity of 40 - 60 $\mu E/m^2s$, with a photoperiod of 16 hours light and 8 hrs dark at 28° C.

[00276] Cotyledon segments (~5 mm) square are isolated from 7 - 10 day old seedlings into liquid M-medium (MS-based medium with 1-5 μM 2,4-D and 1-5 μM Kinetin) in Petri plates. Cut segments are treated with an *Agrobacterium* solution then transferred to semi-solid M-medium and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to M-medium containing 5 mg/l glufosinate-ammonium medium and 500 mg/l carbenicillin (MG5 medium). 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.

[00277] After 3 weeks, callus from the cotyledon segments is isolated and transferred to fresh MG5 medium. The callus is transferred for an additional 3 weeks on MG5 medium. Callus is then transferred to C-medium (MS-based medium containing 10-20 μM NAA and 5-10 μM Kinetin) containing glufosinate-ammonium and carbenicillin, and transferred again to fresh selection medium after 3 weeks. After another 3 weeks the callus tissue is transferred to culture medium lacking plant

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growth regulators for embryogenic callus induction. After 4 - 8 weeks on this medium, embryogenic callus is formed; and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color.

[00278] Larger, well-developed embryos are isolated and transferred to basal medium for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to basal medium for shoot and root development. After 4 - 8 weeks, any well-developed plants are transferred into soil and grown to maturity.

[00279] T_0 plants will be allowed to self-pollinate in the greenhouse to give rise to T_1 seed. T_1 plants (and to the extent enough T_0 plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by *AAD-1* and *PAT* genes in transgenic cotton. Rates of 2,4-D used on T_0 plants will typically use one or two selective rates in the range of 50-400 g ae/ha. T_1 seed will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T_0 and T_1 plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Analysis of protein expression will occur as described in example 9 for *Arabidopsis* and corn.

Example 13 – *Agrobacterium* Transformation of Other Crops

[00280] 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.* Hinchee *et al.* 1988. For *Agrobacterium*-mediated transformation of sorghum, *see e.g.* Zhao *et al.*, 2000. For *Agrobacterium*-mediated transformation of barley, *see e.g.* Tingay *et al.*, 1997. For *Agrobacterium*-mediated transformation of wheat, *see e.g.* Cheng *et al.*, 1997. For *Agrobacterium*-mediated transformation of rice, *see e.g.* Hiei *et al.*, 1997.

Example 14 – Vector Construction and Stacking *AAD-1* With Other Herbicide Resistance Genes

[00281] *AHAS* Vector construction: Whiskers transformation. A corn *AHAS* gene having a mutation coding for asparagine instead of serine at residue 621 of the protein (see US Patent Nos. 5,731,180, 5,767,361 and 5,928,937 to Cyanamid) was received from BASF in plasmid pAC1565. Five

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restriction enzyme recognition sites were removed from the *AHAS* gene without altering the amino acid sequence, and both a multiframe stop codon and a SacI site were added to the 3' end of the gene. A sixth modification of AAT to AAC at amino acid 621 was also completed. The *AHAS* gene was then used to replace the *PAT* gene in pDAB8504 (RB7 MAR/OsAct1/*PAT*/Zmlip/RB7 MAR) to create a whiskers backbone vector containing *AHAS*.

[00282] A short PCR fragment was designed to add the multiframe stop codons and a SacI site at the 3' end of the *AHAS* gene. The sense primer (AHAS 5') and the antisense primer (AHAS 3') were used to amplify the 3' end of the BASF *AHAS* gene to create the 3' AHAS Adapter. The adapter was then ligated into pDAB8504 on an NcoI/SacI fragment replacing the *PAT* gene to create pDAB2210. A unique enzyme, RsrII, was left in the adapter to enable the remainder of the *AHAS* gene to be easily ligated into pDAB2210 (RB7 MAR/OsAct1/*AHAS* Adapter/Zmlip/RB7 MAR) on an NcoI/RsrII digest after the rest of the modifications were completed.

[00283] Modifications to remove the 5 restriction enzyme recognition sites and alter amino acid 621 were done with the QuikChange® Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Changes M1-3, 5, 6 were done in succession with the starting material being pAC1565. M4 was done on pDAB2210. These changes are summarized in **Table 19**.

Table 19.

Modification	Goal	Primers
M1	Remove NcoI site at bp1115 of pAC1565	M1 & M1R
M2	Remove NcoI site at bp1310 of pAC1565	M2 & M2R
M3	Remove HindIII site at bp1993 of pAC1565	M3 & M3R
M4	Modify AA 621 of AHAS gene from AAT to AAC	M4 & M4R
M5	Remove FspI site at bp1677 of pAC1565	M5 & M5R
M6	Remove FspI site at bp1786 of pAC1566	M6 & M6R

[00284] Upon completion of all 6 changes, the gene was reassembled in pDAB2210 by ligating the NcoI/RsrII fragment containing the modified gene from pAC1565 into pDAB2210 to create pDAB2212 (RB7 MAR/OsAct1/*AHAS*/Zmlip/RB7 MAR).

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[00285] Stacking *AAD-1* and *AHAS*. The ZmUbi1/AAD-1/ZmPer5 cassette was removed from pDAB3404 with AscI/SwaI and inserted into pDAB2212 to create the *AAD-1* and *AHAS* Whiskers transformation vector pDAB3415.

[00286] Stacking *AAD-1* and *EPSPS*. A gene encoding a glyphosate tolerant form of the enzyme 5-enolpyruvlyshikimate-3-phosphate synthase (EPSPS) is isolated, *e.g.*, from the soil bacterium *Agrobacterium tumefaciens* strain CP4 and modified for plant-preferred codons (*see, e.g.*, U.S. Patent Nos. 5,804,425 and 5,633,435). Native *EPSPS* genes can also be engineered into glyphosate-tolerant forms, as is described, *e.g.*, in WO9506128, WO 9704103, WO9854330, and U.S. Patent No. 6,040,497. These *EPSPS* genes are preferably fused to a chloroplast transit peptide to allow subcellular targeting of the glyphosate-tolerant EPSPS proteins into the chloroplast (*see, e.g.*, US Patent Nos. 4,940,835, 5,188,642 and 6,063,601), then ligated, for example, into plasmid pDAB2212 to replace the *PAT* gene using standard cloning techniques (*see, e.g.*, Sambrook and Russell2000).The ZmUbi1/*AAD-1*/ZmPer5 cassette is removed from pDAB3404 with AscI/SwaI and inserted into the above vector to create the *AAD-1* and *EPSPS* Whiskers transformation vector.

[00287] *AHAS* Vector construction: Agrobacterium Transformation. A binary vector was constructed containing the *AHAS* selectable marker for use in the Maize Agrobacterium mediated transformation system. Plasmid pDAB2212 was digested with PmeI to isolate the OsAct1/*AHAS*/Z mlip cassette and the fragment ligated into pDAB8546 to create pDAB8548 (RB7 MAR/OsAct1/*AHAS*/Z mlip with Agro backbone).

[00288] The artificial MAR sequence was isolated from pdartMAR with a PmeI digest. The MAR was inserted into pDAB8548 at the MluI (blunted) to create pDAB8549. The resulting base vector, pDAB8549, contained non-identical MAR regions, RB7 MAR 5' and artificial MAR 3'.

[00289] Stacking *AAD-1* and *AHAS*. The ZmUbi1/*AAD-1*/ZmPer5 cassette was isolated from pDAB3404 with a NotI digest and inserted into pDAB8549 to create pDAB2272. This intermediate vector was then transformed into *Agrobacterium* strain LBA4404 containing the pSB1 plasmid (U.S. Patent 5,591,616).

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Example 15 – Further Evidence of Surprising Results: *AAD-1 vs. AAD-2*

[00290] *AAD-2* initial cloning. 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* 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.

[00291] The strain of *Bradyrhizobium japonicum* containing the *AAD-2* 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 150rpm. 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)].

[00292] 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/ul), H2O 21 μl, Taq polymerase 1 ul (2.5 units/ ul). 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.

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[00293] 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* for internal consistency.

[00294] Completion of *AAD-2* Binary Vector. The *AAD-2* 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* gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: *AAD-2*: 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*: 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*.

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[00295] Functional enzyme assay (colorimetric). Colorimetric assays were conducted as previously described in Example 3. Assays were performed with unpurified desalted extracts. Results are reported in **Table 20**.

Table 20. Comparison of the Km and "Vmax" values for the aryloxyalkanoate dioxygenases (AADs) from pDAB3202 (AAD-2) and pDAB3203 [AAD-1 (v1)] with different herbicide substrates:

Enzyme	Compound	Km (μM) ±SE	Vmax (A510 units)	Km/Vmax (arbitrary units)
AAD-2	2,4-D	423 (±1)	0.86	2.03
AAD-1 (v1)	2,4-D	97 (±21)	0.11	1.16

Notes: Assays were performed in MOPS pH 6.75 + 1 mM α-ketoglutarate + 0.1 mM Na ascorbate + 0.1 mM Fe2+ and the released phenols colorimetrically detected using 4-aminoantipyrine/ferricyanide.

[00296] Current data indicate that AAD-2 is an S-enantiomer-specific enzyme whereas AAD-1 is R-enantiomer-specific. Therefore dichlorprop K_m values were calculated based on the single enantiomer concentration rather than the total racemic mix used.

[00297] The fitted curves from the data are shown in **Figure 11,** which illustrates the relative rates of herbicide degradation for the various substrates by the two enzymes (AAD-2 vs. AAD-1). The concentrations of AADs in each extract are approximately equivalent, which suggests that AAD-2 is a better degrader of 2,4-D than in AAD-1. However, these *in vitro* results are in surprising contrast to the *in planta* findings reported below, where AAD-1 is surprisingly much superior to AAD-2 in regards to 2,4-D resistance.

[00298] Evaluation of transformed Arabidopsis. Freshly harvested T_1 seed transformed with a native [*AAD-1 (v2)*], plant optimized [*AAD-1 (v3)*], or native *AAD-2* 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.

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[00299] 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).

[00300] Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200g ai/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 ml / tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5°C, 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural + supplemental light) at least 1 day prior to testing for the ability of *AAD-1 (v3)*, *AAD-1 (v2)*,or *AAD-2* to provide phenoxy auxin herbicide resistance.

[00301] 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).

[00302] 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).

Results of testing.

[00303] 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

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frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (**Table 14**). A small lot of *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). Surpisingly, *Arabidopsis* transformations using the *AAD-2* 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 14**). Transformation frequency for native *AAD-2* was only 6% of that of *AAD-1 (v2)*.

Table 14: Selection of AAD-1 v3 (plant optimized), or AAD-1 v2 (native), or AAD-2 (native)-transformed T1 individual plants using glufosinate and 2,4-D.

Selection agent	Gene	Total seed sown and screened	Number of resistant T_1	Transformation/ Selection rate	Selection rate range	% of selected plants expressing PAT[3]
Glufosinate[1]	*AAD-1 v3*	400,500	493	0.12%	0.05 to 0.23%	97%
2,4-D[2]	*AAD-1 v3*	70,000	53	0.08%	0.07 to 0.08%	96%
Glufosinate[1]	*AAD-1 v2*	84,000	278	0.33%	0.33%	nd[4]
Glufosinate[1]	*AAD-2*	1,301,500	228	0.018%	0.07 to 0.09%	100%

[1]Glufosinate selection scheme: 280g ai/ha glufosinate applied 5-6 + 10 DAP
[2]2,4-D selection scheme: 50 g ai/ha 2,4-D applied 5-7 + 10-14 DAP
[3]PAT protein expression determined by PAT ELISA strips
[4]nd, not determined

[00304] T_1 plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. **Table 15** compares the response of *AAD-1 (v2)* with *AAD-1 (v3)* and *AAD-2* genes to impart 2,4-D resistance to Arabidopsis T_1 transformants. All genes did provide some significant 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T_1 *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in **Table 15**

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simply to demonstrate the significant difference between the plants transformed with *AAD-1 (v2)*, *AAD-1 (v3)*, or *AAD-2* versus the wildtype or *PAT/Cry1F* transformed controls.

[00305] Surprisingly, *AAD-2* transformants were far less resistant to 2,4-D than either *AAD-1 (v2)* or *AAD-1 (v3)* genes (**Table 15**) both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83%. Conversely, 56% (45 of 80) *AAD-1 (v2)*-transformed T_1 plants survived 200 g ae/ha 2,4-D uninjured (population injury average = 34%), and >73% (11 of 15) *AAD-1 (v3)* T_1 plants were uninjured (population injury average = 14%). *See* **Figure 12**.

[00306] These results are unexpected given that *in vitro* comparison of native *AAD-1* and *AAD-2* indicated 2,4-D was better degraded by *AAD-2*. *AAD-2* is expressed in individual T_1 plants to varying levels with the anticipated size; however, little protection form 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 13**. These data corroborate earlier findings that make the functional expression of AAD-1 *in planta* for imparting herbicide resistance to 2,4-D and AOPP herbicides is unexpected and not obvious.

Example 16 – Preplant Burndown Applications

[00307] This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

[00308] 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

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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* transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone + 2,4-D or glufosinate + 2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the *AAD-1* gene if stably transformed.

Example 17 – In-Crop Use of Phenoxy Auxins Herbicides in *AAD-1* Only Transformed Soybeans, Cotton, and Other Dicot Crops

[00309] *AAD-1* 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

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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-transformed dicot crop (*i.e.*, soybean or cotton) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (*see* weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00310] 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* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

[00311] 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* example 16); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D. Dicamba (and other herbicies) can still be used for the subsequent control of *AAD-1*-transformed dicot crop volunteers.

[00312] 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* gene if

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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* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 18 - In-Crop Use of Phenoxy Auxins and AOPP Herbicides in *AAD-1* Only Transformed Corn, Rice, And Other Monocot Crops

[00313] In an analogous fashion, transformation of grass crops (such as, but not limited to, corn, rice, wheat, and barley) with *AAD-1* 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*-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*-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* transformed monocot crop (*e.g.*, corn and rice) that would control most

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agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

[00314] 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* HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

[00315] 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* 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* transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

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Example 19 – *AAD-1* stacked with glyphosate tolerance trait in any crop

[00316] 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. 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* 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*, 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* 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.

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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* + 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.

[00317] 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* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20 - *AAD-1* Stacked with Glufosinate Tolerance Trait in Any Crop

[00318] 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

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24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agriliance, 2003).

[00319] By stacking *AAD-1* 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*, 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* 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.

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[00320] 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* transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (*e.g.*, cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21 - *AAD-1* Stacked with *AHAS* Trait in Any Crop

[00321] Imidazolinone herbicide tolerance (*AHAS, et al.*) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (*e.g.*, cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (*e.g.*, imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like *AHAS et al.* Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) for which many weeds have developed resistance to (Heap, 2004). By stacking *AAD-1* 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*, 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

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options can be envisioned where *AAD-1* 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.

[00322] 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* 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

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Protection Guide from Agriliance (2003). Each alternative herbicide enabled for use in HTCs by *AAD-1*, whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

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Abstract

[00323] 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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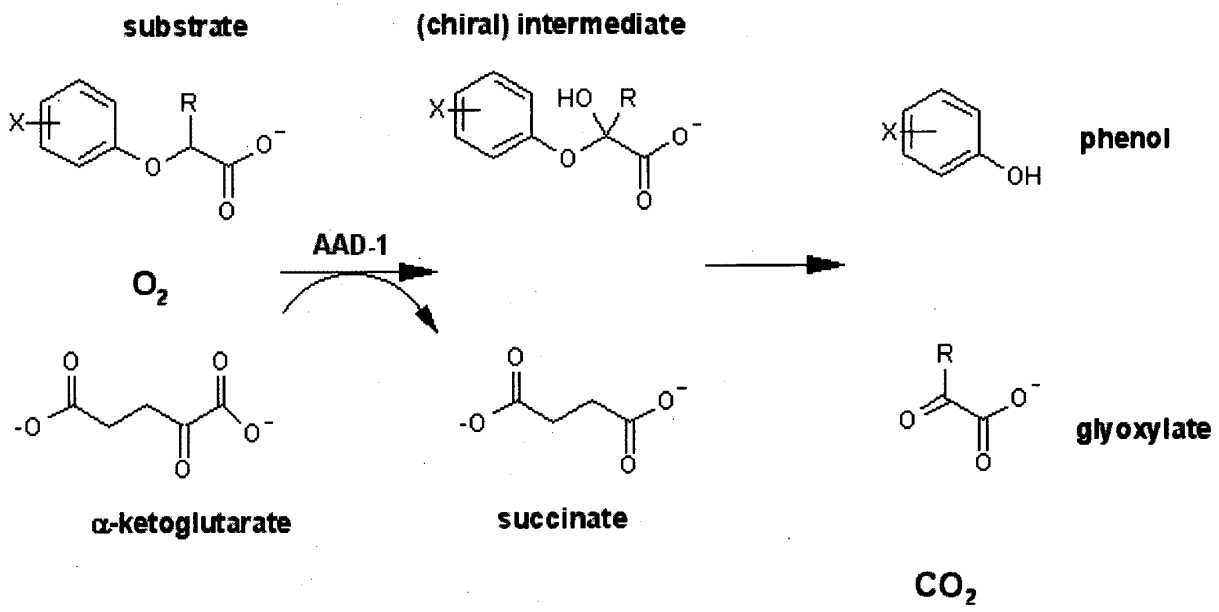
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Figure 1: General scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.



- Addition of O_2 is *stereospecific*
- Breakdown of intermediate to phenol + glyoxylate is spontaneous

Figure 2: Representative data showing the loss of herbicidal activity from a 2,4-D solution treated with heterologously-expressed AAD-1 (v1) enzyme extract. EM2 is the background culture media and the pET vector control extract has no *AAD-1 (v1)* gene to express.

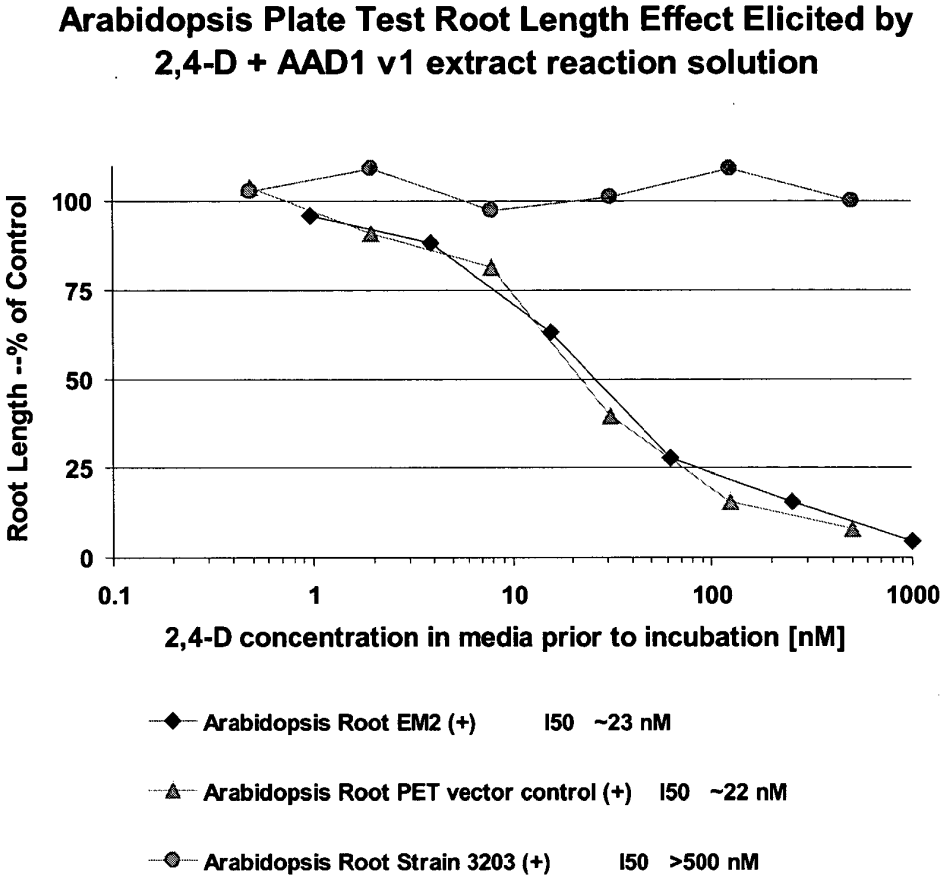


Figure 3: Representative data showing the loss of herbicidal activity from a haloxyfop solution treated with heterologously-expressed AAD-1 (v1) enzyme extract. pET vector control extract has no *AAD-1 (v1)* gene to express.

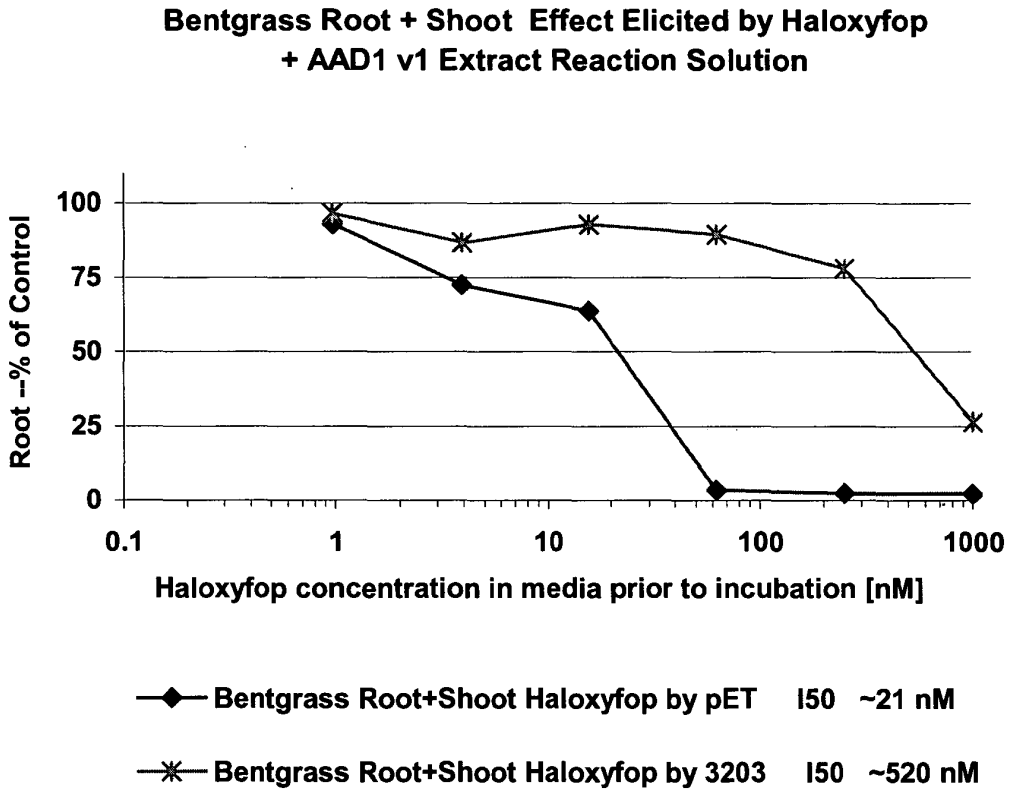


Figure 4: Anticipated phenols produced from various herbicides catalyzed by AAD-1.

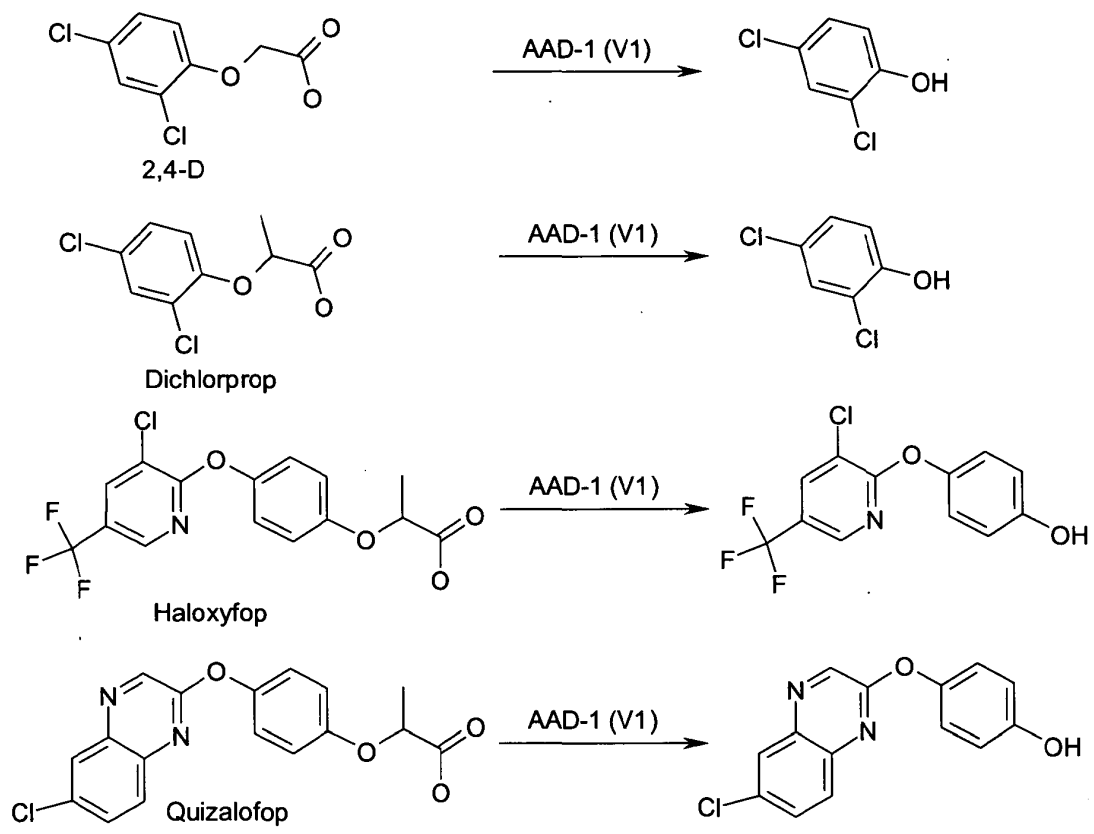


Figure 5: Activity of recombinant AAD-1 (v1) expressed in E. coli. **2,4-dichlorophenol production monitored by colorimetric detection at 510 nm.** Varying amounts of extracts from *E. coli* expressing a plasmid for *AAD-1 (v1)* or a control plasmid were assayed using 1 mM 2,4-D as substrate.

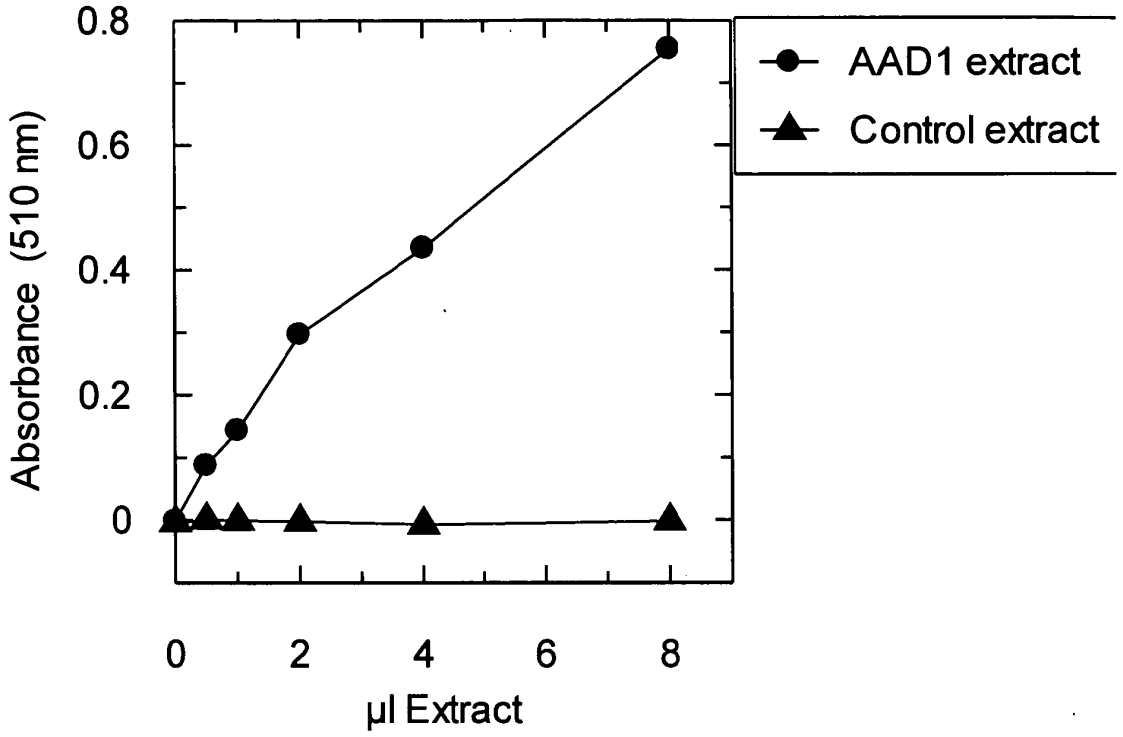
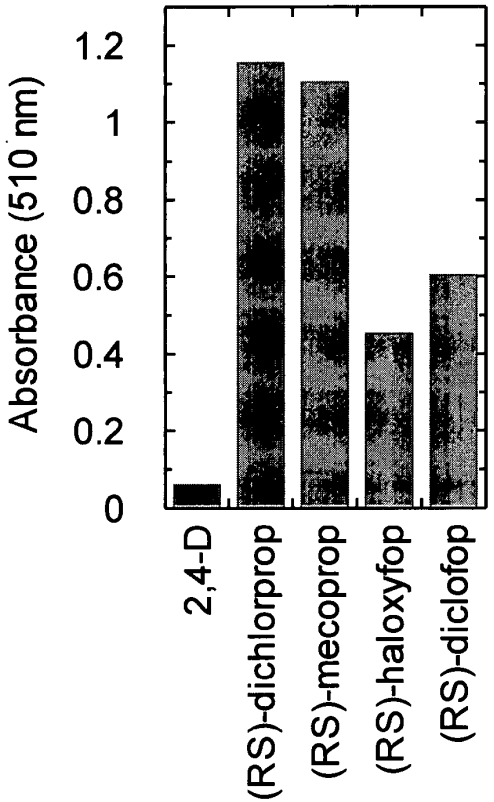


Figure 6: Activity of recombinant AAD-1 (v1) expressed in E. coli. **Phenol production by AAD-1 (v1) extract from various herbicide substrates:** Substrates were assayed at 0.5 mM for 15 min in 25 mM MOPS pH 6.8, 200 µM Fe^{2+}, 200 µM Na ascorbate, 1 mM α-ketoglutarate using 4 µl *E. coli* extract.

6A.



6B.

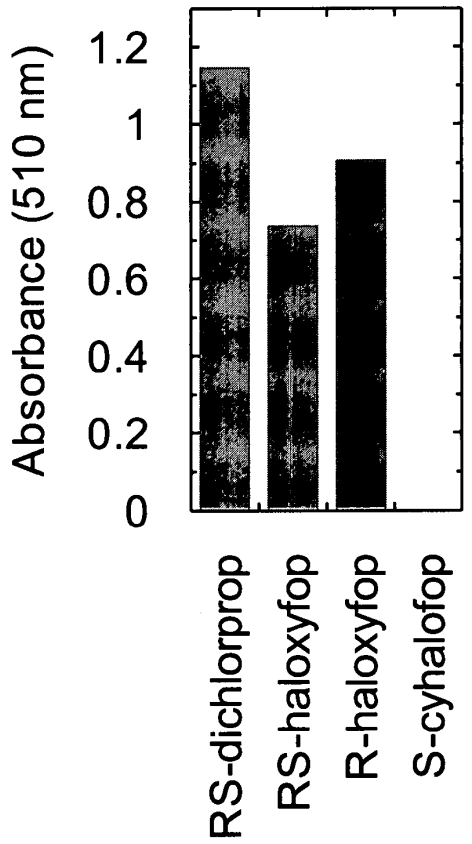


Figure 7: **Response of AAD-1 (v1) reaction rate to substrate concentration for four herbicide substrates.** Curves were fitted to the experimental data using Grafit and the Michaelis-Menten equation. The derived kinetic constants are shown in Table 9.

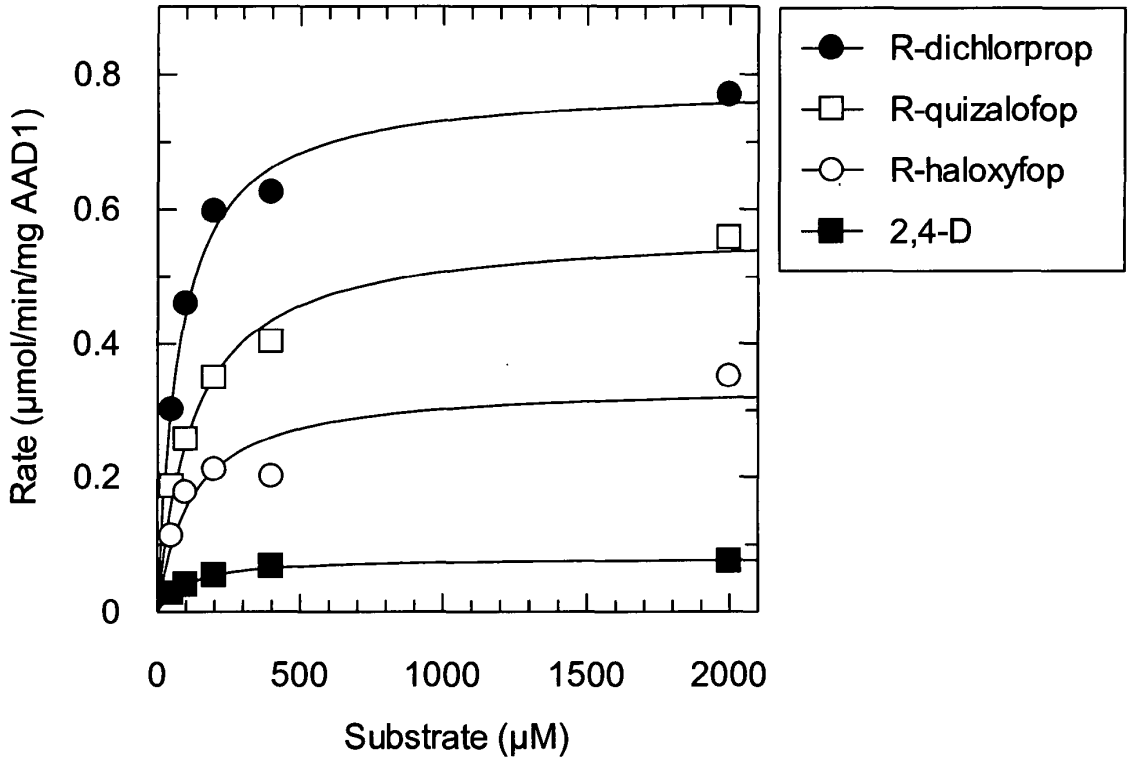
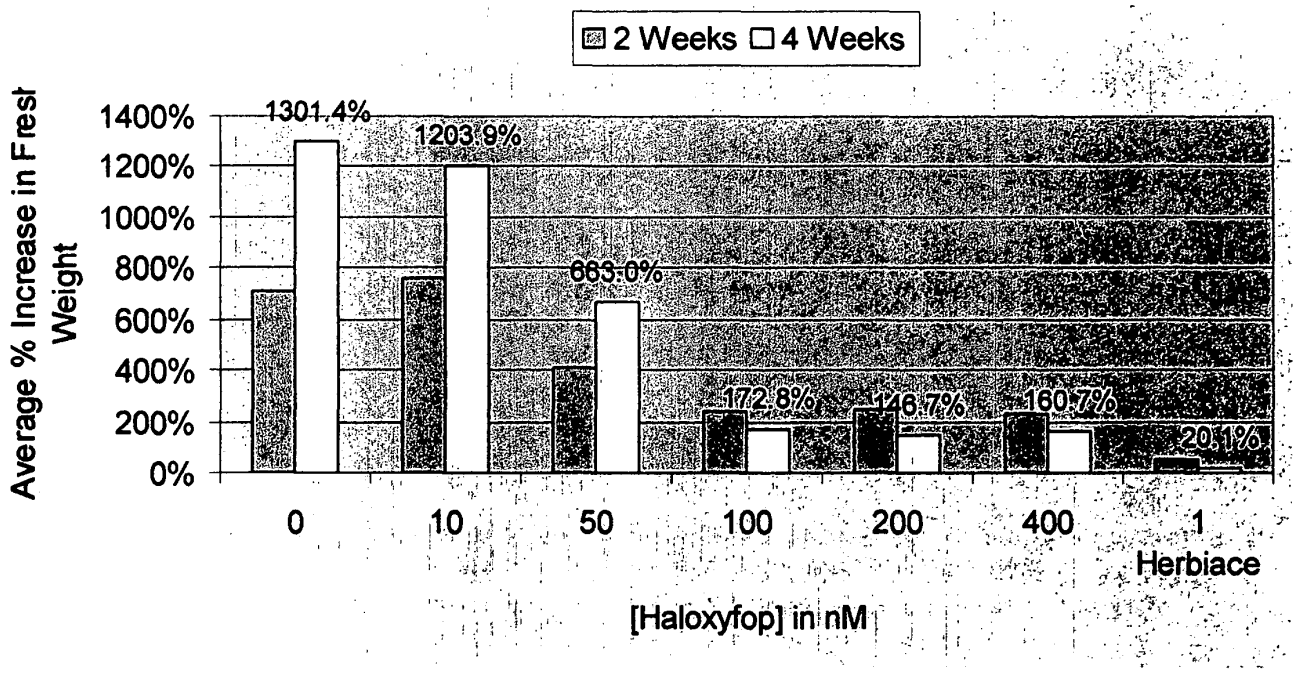
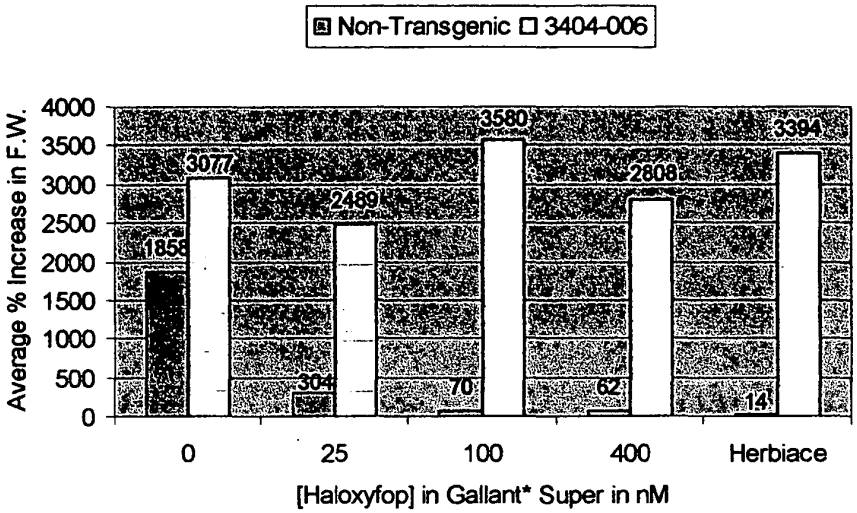


Figure 8: Dose response of callused maize suspensions to R-haloxyfop after 4 weeks.



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Figure 9. *AAD-1 (v3)*-transformed corn callus response to haloxyfop.



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Figure 10. Response 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.

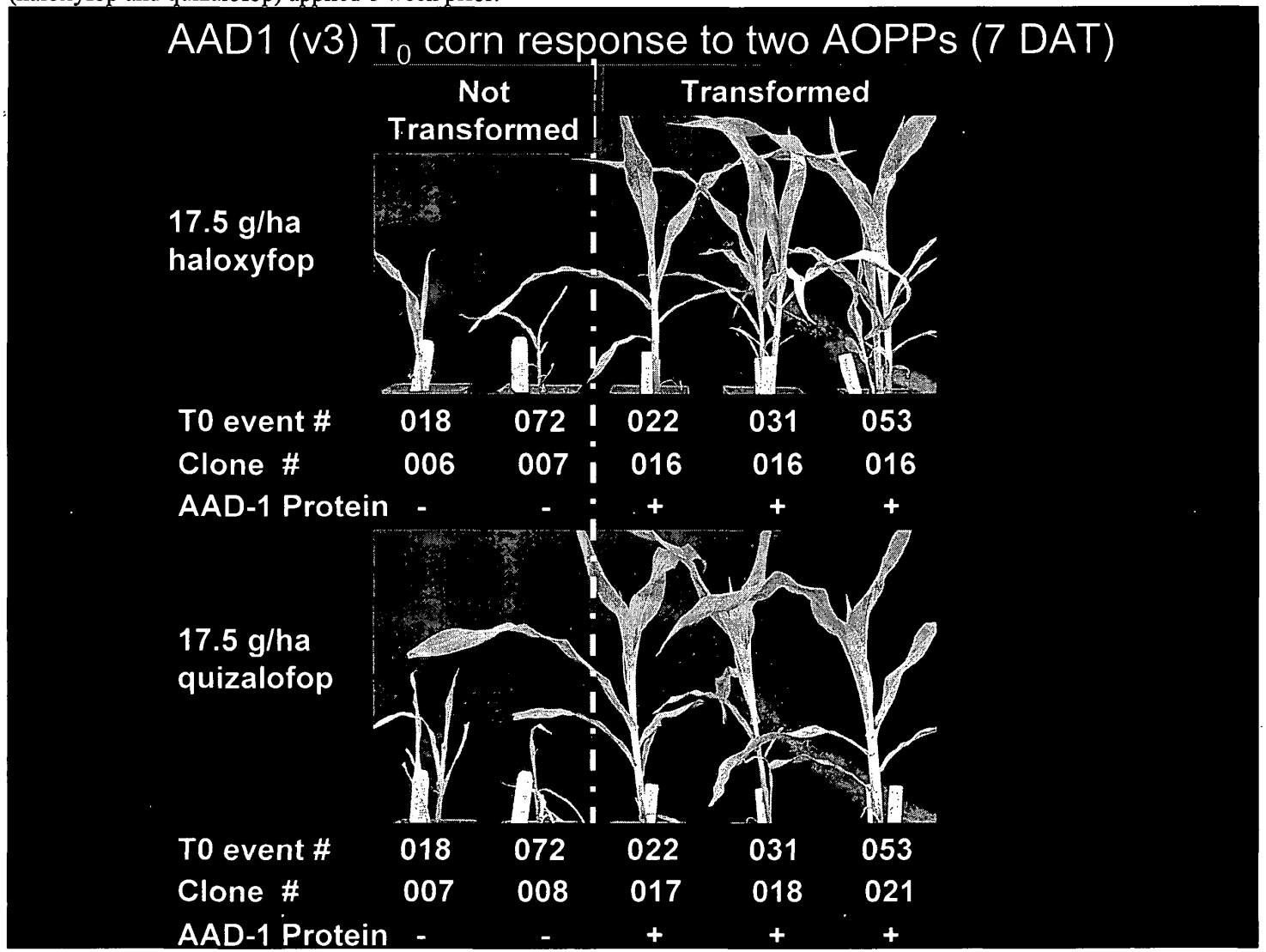


Figure 11. Fitted curves showing relative activities on 2,4-D by AAD-1 (v1) and AAD-2. Concentrations of AADs in each extract are approximately equivalent

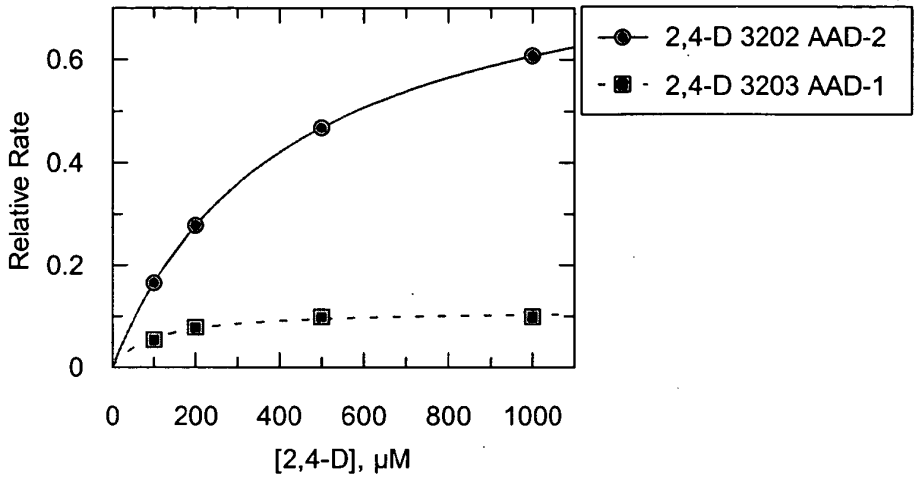


Figure 12. Dose response of AAD-1 v3 (plant optimized), AAD-1 v2 (native), or AAD-2 (native)-transformed T_1 Arabidopsis to a range of 2,4-D rates applied postemergence. Each pot represents an individual transformation event within each gene T1 family. The relative protective capability from 2,4-D injury of individual genes is plant optimized *AAD-1 (v3)* > native *AAD-1 (v2)* > native *AAD-2*.

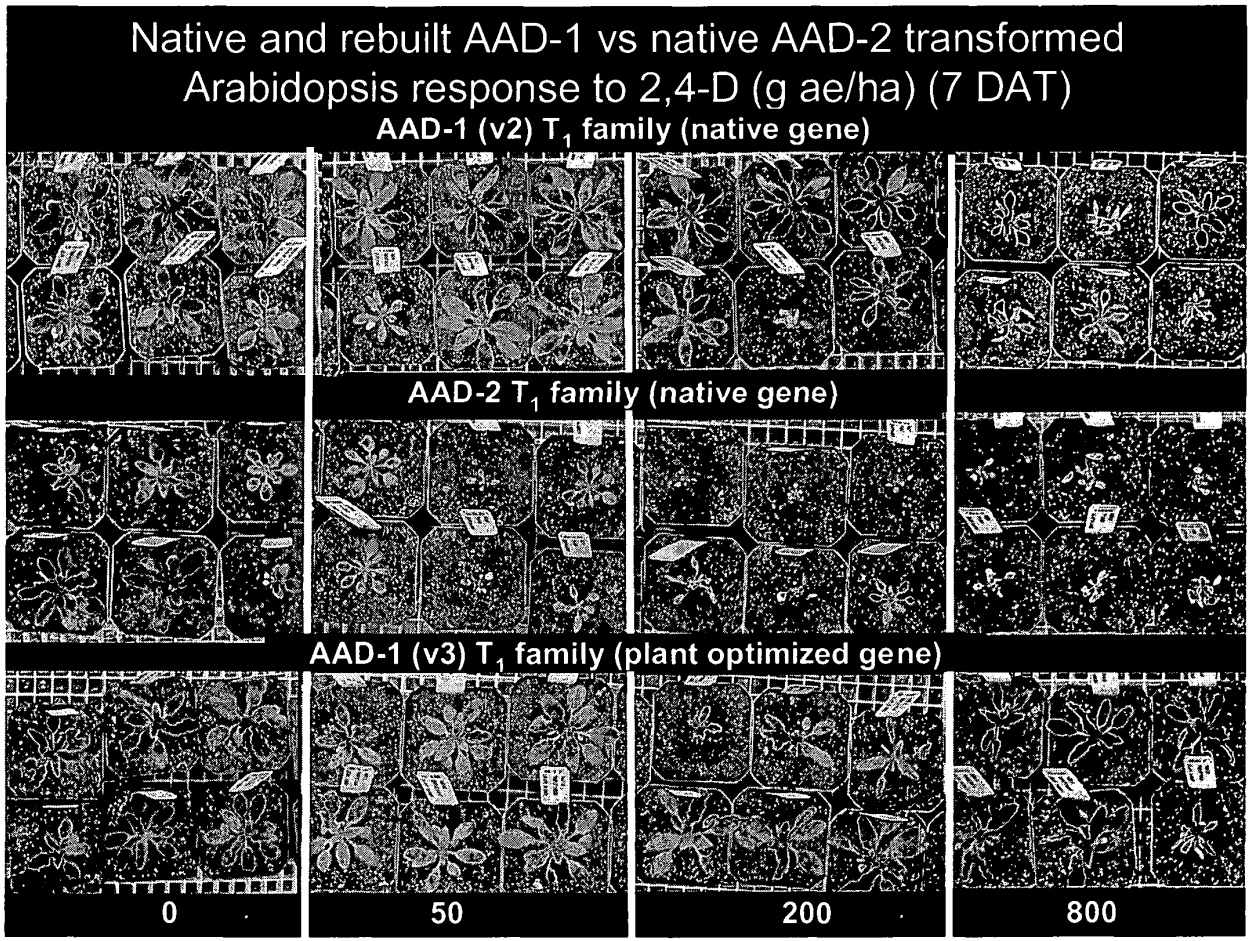
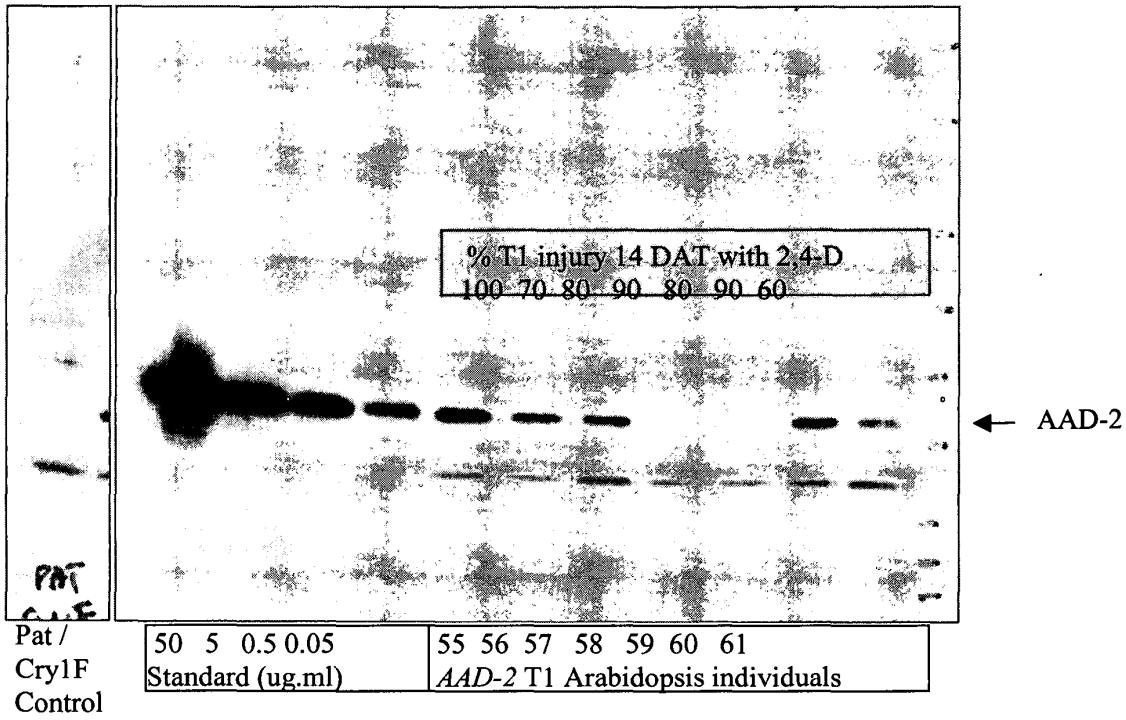


Figure 13. Western blot analysis of individual native *AAD-2*-transformed T1 Arabidopsis plants. This demonstrates plants expressing the AAD-2 protein suffering severe injury from 200 g ae/ha 2,4-D treatments, which normally causes little injury to native *AAD-1 (v2)* or plant optimized *AAD-1 (v3)*-transformed Arabidopsis. AAD-2 protein is identified on the gel. Several background bands were detected in AAD-2 transformed and Pat/Cry1F transformed samples.



SEQ ID No. 1:

AAD-1 forward primer:

TCTAGAAGGAGATATACCATGCATGCTGCACTGTCCCCCCTCTCCCAGCG

SEQ ID No. 2:

AAD-1 reverse primer:

CTCGAGTTACTAGCGCGCCGGGCGCACGCCACCGACCG

SEQ ID No. 3:

AAD-1 (v1) primary sequence: (primer linkers are highlighted and underlined)

TCTAGAAGGAGATATACCATGCATGCTGCACTGTCCCCCCTCTCCCAGCGCTTTGAGCGCA
TCGCGGTCCAGCCGCTGACCGGCGTCCTGGGCGCCGAGATCACCGGCGTCGACCTGCGC
GAGCCGCTCGACGACAGCACCTGGAACGAAATCCTCGACGCGTTCCACACTTACCAGGTCA
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SEQ ID No. 4:

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SEQ ID No. 5:

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CACCAGATTTGACTTCACTTGCCGTGTGAGGTGGAAGAAAGACCAAGTCCTTGTCTGGGAC
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CACCACAGTTGGTGGAGTTAGGCCTGCCCGCTGAGTAGTTAGCTTAATCACCTAGAGCTC

SEQ ID No. 6:

RdpA(ncoI) 5" primer

CCC ATG GCT GCT GCA CTG TCC CCC CTC TCC

SEQ ID No. 7:

3'saci primer

GAG CTC ACT AGC GCG CCG GGC GCA CGC CAC CGA

SEQ ID No. 8:

BstEII/ Del NotI 5' primer

TGG TGG TGA CCC ATC CGG GCA GCG GCT GCA AGG GCC

SEQ ID No. 9:

AAD-1 v1 Translation (primary protein sequence)

MHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTY
QVIYFPGQAITNEQHIAFSRRFGPVDPVPLLKSIEGYPEVQMIRREANESGRV
IGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQ
ATIEGLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVT
HPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQFLYEHATRFDFTCRVRWK
KDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR*

SEQ ID No. 10:

AAD-1 v2 Translation (differences from v1 are highlighted)

MAAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTY
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IGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTMQ
ATIEGLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVVT
HPGSGCKGLYVNQVYCQRIEGMTDAESKPLLQFLYEHATRFDFTCRVRWK
KDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR*

SEQ ID No. 11:

AAD-1 v3 Translation

MATHAALSPLSQRFERIAVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHT
YQVIYFPGQAITNEQHIAFSRRFGPVDPVPLLKSIEGYPEVQMIRREANESGR
VIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSMYTAWETLSPTM
QATIEGLNVVHSATRVFGSLYQAQNRRFSNTSVKVMDVDAGDRETVHPLVV
THPGSGRKGLYVNQVYCQRIEGMTDAESKPLLQFLYEHATRFDFTCRVRWK
KDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVGGVRPAR*

SEQ ID No. 12

AAD-2 nucleotide sequence

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TCACCGACGAGCAGCAGATGGCTTTCGCGCTGAACTTCGGCCAGCGCGA
GGACGCGCGCGGCGGCACGGTCACCAAGGAGAAGGACTACCGGCTGCA
ATCCGGCCTGAACGACGTCTCCAATCTCGGCAAGGACGGCAAGCCGCTG
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CGCGCCGCCTATGACGCGCTCGACGACGAGACCAAGGCCGAAATCGAG
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TCACCGAGTACACCGACGAAGAGAAGCAGATGTTCAAGCCGGTCCTGCAA
CGCCTCGTGCGCACCCATCCGGTCCACCGCCGCAAGTCGCTGTATCTCT
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TGCTGTTGCGCGATCTCAACGAGCACGCGACGCAGCCGGAATTCGTCTA
CGTCCACAAATGGAAGCTGCATGACCTCGTGATGTGGGACAACCGCCAG
ACCATGCACCGCGTCCGCCGCTACGACCAGTCCCAGCCCCGCGACATGC
GCCGCGCGACGGTGGCGGGGACGGAGCCGACGGTGCAGCAGCAGGCG
GCGGAGTAG

SEQ ID No. 13

AAD-2 translation

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DSRTHLFNLGNCLWHSDSSFRPIPAKFSLLSARVVNPTGGNTEFADMRAAY
DALDDETKAEIEDLVCEHSLMYSRGSLGFTEYTDEEKQMFKPVLQRLVRTH
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SEQ ID No. 14

brjap 5'(spel)

ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T

SEQ ID No. 15

br jap 3' (xhol)

TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC

SEQ ID No. 16

M13 Forward

GTA AAA CGA CGG CCA GT

SEQ ID NO:17

M13 Reverse

CAG GAA ACA GCT ATG AC

SEQ ID No. 18

NcoI of Brady

TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT

SEQ ID No. 19

SacI of Brady

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Bayer EX1005-0406 (1 of 2)

RAW SEQUENCE LISTING DATE: 11/08/2006
PATENT APPLICATION: **US/11/587,893** TIME: 11:53:54

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Output Set: **N:\CRF4\11082006\K587893.raw**

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67 gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttccacac ttaccaggtc 180
69 atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc 240
71 ggccccgtcg atcccgtgcc cctgctcaag agcatcgaag ggtatccaga ggtgcagatg 300
73 atccgccgcg aagccaacga aagcgggcgt gtgatcggtg aygactggca caccgacagc 360
75 accttcctgg acgcaccgac ggccgccgtg gtgatgcgcg cgatcgacgt gcccgagcat 420
77 ggcggcgaca ccggttttct gagcatgtac accgcgtggg agacgctgtc gcccaccatg 480
79 caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggctcgctc 540
81 taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg 600
83 ggcgaccgtg aaaccgtgca ccccctggtg gtgacccatc cgggcagcgg ccgcaagggc 660
85 ctgtacgtga accaggtcta ttgccagcgc atcgagggca tgaccgatgc cgaaagcaaa 720
87 ccgctgctgc agttcctgta cgagcatgcg acacggttcg atttcacctg ccgcgtgcgc 780
89 tggaagaagg accaggtcct ggtctgggac aacctgtgca cgatgcaccg ggccgtaccc 840
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99 <213> ORGANISM: Artificial Sequence
101 <220> FEATURE:
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124 tgcccctgct caagagcatc gaagggtatc cagaggtgca gatgatccgc cgcgaagcca 300
126 acgaaagcgg gcgtgtgatc ggtgatgact ggcacaccga cagcaccttc ctggacgcac 360
128 cgccggccgc cgtggtgatg cgcgcgatcg acgtgcccga gcatggcggc gacaccggtt 420
130 ttctgagcat gtacaccgcg tgggagacgc tgtcgcccac catgcaggcc accatcgaag 480
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148 <211> LENGTH: 919
149 <212> TYPE: DNA

Bayer EX1005-0407 (1 of 2)

RAW SEQUENCE LISTING DATE: 11/08/2006
PATENT APPLICATION: **US/11/587,893** TIME: 11:53:54

Input Set : **A:\DAS-115XC1.ST25.txt**
Output Set: **N:\CRF4\11082006\K587893.raw**

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169 <223> OTHER INFORMATION: primer linker
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176 acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcatc tactttcctg 180
178 gccaagcaat caccaatgag cagcacattg cattctcaag aaggtttgga ccagttgatc 240
180 cagtgcctct tctcaagagc attgaaggct atccagaggt tcagatgatc cgcagagaag 300
182 ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttccttgatg 360
184 cacctccagc tgctgttgtg atgagggcca tagatgttcc tgagcatggc ggagacactg 420
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192 cagtccatcc cttggttgtg actcatcctg gctctggaag gaaaggcctt tatgtgaatc 660
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196 tcctctatga gcatgccacc agatttgact tcacttgccg tgtgaggtgg aagaaagacc 780
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Bayer EX1005-0408 (1 of 2)

RAW SEQUENCE LISTING DATE: 11/08/2006
PATENT APPLICATION: **US/11/587,893** TIME: 11:53:54

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Output Set: **N:\CRF4\11082006\K587893.raw**

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            20                  25                  30
Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
        35                  40                  45
Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
    50                  55                  60
Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
65                  70                  75                  80
Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
                85                  90                  95
Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
            100                 105                 110
Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
        115                 120                 125
Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
    130                 135                 140
Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
145                 150                 155                 160
Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
                165                 170                 175
Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
            180                 185                 190
Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
        195                 200                 205
Gly Ser Gly Arg Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220
Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
225                 230                 235                 240
Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
                245                 250                 255
Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
            260                 265                 270
Val Pro Asp Tyr Ala Gly Lys Phe Arg Tyr Leu Thr Arg Thr Thr Val
        275                 280                 285
Gly Gly Val Arg Pro Ala Arg
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Bayer EX1005-0409 (1 of 2)

RAW SEQUENCE LISTING DATE: 11/08/2006
PATENT APPLICATION: **US/11/587,893** TIME: 11:53:54

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Output Set: **N:\CRF4\11082006\K587893.raw**

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350             20                  25                  30
353 Leu Arg Glu Pro Leu Asp Asp Ser Thr Trp Asn Glu Ile Leu Asp Ala
354         35                  40                  45
357 Phe His Thr Tyr Gln Val Ile Tyr Phe Pro Gly Gln Ala Ile Thr Asn
358     50                  55                  60
361 Glu Gln His Ile Ala Phe Ser Arg Arg Phe Gly Pro Val Asp Pro Val
362 65                  70                  75                  80
365 Pro Leu Leu Lys Ser Ile Glu Gly Tyr Pro Glu Val Gln Met Ile Arg
366                 85                  90                  95
369 Arg Glu Ala Asn Glu Ser Gly Arg Val Ile Gly Asp Asp Trp His Thr
370             100                 105                 110
373 Asp Ser Thr Phe Leu Asp Ala Pro Pro Ala Ala Val Val Met Arg Ala
374         115                 120                 125
377 Ile Asp Val Pro Glu His Gly Gly Asp Thr Gly Phe Leu Ser Met Tyr
378     130                 135                 140
381 Thr Ala Trp Glu Thr Leu Ser Pro Thr Met Gln Ala Thr Ile Glu Gly
382 145                 150                 155                 160
385 Leu Asn Val Val His Ser Ala Thr Arg Val Phe Gly Ser Leu Tyr Gln
386                 165                 170                 175
389 Ala Gln Asn Arg Arg Phe Ser Asn Thr Ser Val Lys Val Met Asp Val
390             180                 185                 190
393 Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro
394         195                 200                 205
397 Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
398     210                 215                 220
401 Ile Glu Gly Met Thr Asp Ala Glu Ser Lys Pro Leu Leu Gln Phe Leu
402 225                 230                 235                 240
405 Tyr Glu His Ala Thr Arg Phe Asp Phe Thr Cys Arg Val Arg Trp Lys
406                 245                 250                 255
409 Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg Ala
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Bayer EX1005-0410 (1 of 2)

VERIFICATION SUMMARY DATE: 11/08/2006
PATENT APPLICATION: **US/11/587,893** TIME: 11:53:55

Input Set : **A:\DAS-115XC1.ST25.txt**
Output Set: **N:\CRF4\11082006\K587893.raw**

L:12 M:270 C: Current Application Number differs, Replaced Current Application No
L:12 M:271 C: Current Filing Date differs, Replaced Current Filing Date

Bayer EX1005-0411 (1 of 2)

PTO/SB/08A (08-03)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	11/587,893
Filing Date	October 27, 2006
First Named Inventor	Terry Wright
Art Unit	Not Yet Known
Examiner Name	Not Yet Known
Attorney Docket Number	DAS-115XC1

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1	US- 5,273,894	12/28/1993	Strauch et al.	(Please review all)
	2	US- 5,561,236	10/01/1996	Leemans et al.	(Please review all)
	3	US- 5,608,147	03/04/1997	Kaphammer	(Please review all)
	4	US- 5,637,489	06/10/1997	Strauch et al.	(Please review all)
	5	US- 5,646,024	07/08/1997	Leemans et al.	(Please review all)
	6	US- 5,648,477	07/15/1997	Leemans et al.	(Please review all)
	7	US- 5,879,903	03/09/1999	Strauch et al.	(Please review all)
	8	US- 6,153,401	11/28/2000	Streber et al.	(Please review all)
	9	US- 7,112,665	09/26/2001	Leemans et al.	(Please review all)
		US-			
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FOREIGN PATENT DOCUMENTS

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Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 2 of 2

Complete if Known	
Application Number	11/587,893
Filing Date	October 27, 2006
First Named Inventor	Terry Wright
Art Unit	Not Yet Known
Examiner Name	Not Yet Known
Attorney Docket Number	DAS-115XC1

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	10	GENBANK Accession No. M16730.	
	11	HORVATH, M. et al., "Isolation and characterization of a 2-(2,4-dichlorophenoxy)propionic acid-degrading...", Appl. Microbiol Biotechnol.,1990, pgs.33;213-216. (Abstract only)	
	12	KOHLER, H.P.E., Sphingobium herbicidovorans MH: a versatile phenoxyalkanoic acid...;" J. Ind Microbiol and Biotech; 1999; pgs 23:336-340. (Abstract only)	
	13	LYON, B.R. et al.,"Expression of a bacterial gene in transgenic tobacco confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid" Transgenic Res.1989,pgs.33:166-169.	
	14	LYON, B.R. et al.,"Cotton plants transformed with a bacterial degradation gene are protected from accidental..."; Transgenic Res.; 1993; pgs2:166-169. (Abstract only)	
	15	SMEJKAL, C.W. et al., "Substrate specificity of chlorophenoxyalkanoic acid-degrading bacteria..."; Biol. Fertil., 2001; pgs 33:507-513.	
	16	STREBER, W. et al., "Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D"; Bio/Technology; 1989; pgs 7:811-816. (Abstract only)	
	17	STREBER, W.R. et al., "Analysis, cloning, and high-level expression of 2,4-dichlorophenixyacetic monooxygenase gene..."; J. Bacteriol; 1987; pgs 169:2950-2955.	
	18	WESTENDORF, A. et al., "The two enantiospecific dichlorprop/alpha-ketoglutarate-dioxygenases..."; Microbiol.Res.; 2002; pgs 157:317-22. (Abstract only)	
	19	WESTENDORF, A., "Purification and characterization of the enantiospecific dioxygenases from Delftia acidovorans..."; Acta Biotechnol.; 2003; pgs23:3-17.	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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Display GenBank Show 5 Send to Hide: sequence all but gene, CDS and mRNA features
Range: from begin to end Reverse complemented strand Features: + Refresh

1: M16730. Reports A.eutrophus 2,4-d...[gi:141976] Links

Comment Features Sequence

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            Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
            Burkholderiaceae; Cupriavidus.
REFERENCE   1  (bases 1 to 2058)
  AUTHORS   Streber,W.R., Timmis,K.N. and Zenk,M.H.
  TITLE     Analysis, cloning, and high-level expression of
            2,4-dichlorophenoxyacetate monooxygenase gene tfdA of Alcaligenes
            eutrophus JMP134
  JOURNAL   J. Bacteriol. 169 (7), 2950-2955 (1987)
   PUBMED   3036764
COMMENT     Original source text: A.eutrophus (strain JMP134) DNA, clone pJP4.
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      661 aactcggctg ctctctcgtc tgctggaaca tcttcaggcg cgctgagccg tctttttgaa
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     721 acagtctctt agaaaaggag caaaaaagtg agcgtcgtcg caaatcccct tcatcctctt
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     901 caggatcagc agatcgcctt cgcgcgcaat ttcgggccac tcgaaggcgg tttcatcaag
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    1321 ccggtcaact ggccgctggt tcgaacccac gccggctccg ggcgcaagtt tctcttcatc
    1381 ggcgcgcacg cgagccacgt cgaaggcctt ccggtggccg aaggccggat gctgcttgcg
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    1801 ctcgaaatga cgctggccgc gaaccgcgcc ggcatcgttg cggtgccttt gaactggcat
    1861 gcgaaccggg acgagatcgc cttcatcctc gaggactgca aagcgcgtgt gctcgtcgcg
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    1981 gccgcgtcgc cgcccgagat ccggcaggcc tatcggctgt ccgatgcgtc gtgcacggcg
    2041 aacccgggca cggtcgac
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1: Appl Microbiol Biotechnol. 1990 May;33(2):213-6.

Links

Isolation and characterization of a 2-(2,4-dichlorophenoxy) propionic acid-degrading soil bacterium.

Horvath M, Ditzelmüller G, Loidl M, Streichsbier F.

Abteilung für Mikrobielle Ökologie und Methodik am Institut für Biochemische Technologie und Mikrobiologie, Technische Universität Wien, Vienna, Austria.

The 2-(2,4-dichlorphenoxy)propionic acid (2,4-DP)-degrading bacterial strain MH was isolated after numerous subcultivations of a mixed culture obtained by soil-column enrichment and finally identified as Flavobacterium sp. Growth of this strain was supported by 2,4-DP (maximum specific growth rate 0.2 h-1) as well as by 2,4-dichlorophenoxyacetic acid (2,4-D), 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB), and 2-(4-chloro-2-methylphenoxy)propionic acid (MCPP) as sole sources of carbon and energy under aerobic conditions. 2,4-DP-Grown cells (10(8) of strain MH degraded 2,4-dichlorophenoxyalkanoic acids, 2,4-dichlorophenol (2,4-DCP), and 4-chlorophenol at rates in the range of 30 nmol/h. Preliminary investigations indicate that cleavage of 2,4-DP results in 2,4-DCP, which is further mineralized via ortho-hydroxylation and ortho-cleavage of the resulting 3,5-dichlorocatechol.

PMID: 1366457 [PubMed - indexed for MEDLINE]

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Characterisation of bacterial cultures enriched on the chlorophenoxyalkanoic acid herbicides 4-(2,4-dichlorophenoxy) butyric acid and 4-(4-chloro-2-methylphenoxy) b[illegible] [illegible] 2003]

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Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(RS)-2-(2,4-dichlorophenoxy)propanoic acid] by Sphingomonas herbicidovorans MH. [J Bacteriol. 1998]

A rapid method to screen degradation ability in chlorophenoxyalkanoic acid herbicide-degrading bacteria. [Lett Appl Microbiol. 2001]

Natural selection for 2,4,5-trichlorophenoxyacetic acid mineralizing bacteria in agent orange contaminated soil. [Biodegradation. 2005]

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1: J Ind Microbiol Biotechnol. 1999 Oct;23(4-5):336-340.

Sphingomonas herbicidovorans MH: a versatile phenoxyalkanoic acid herbicide degrader.

Kohler HP.

EAWAG, Swiss Federal Institute for Environmental Sciences and Technology, Ueberlandstrasse 133, CH-8600 Dübendorf, Switzerland.

Sphingomonas herbicidovorans MH was isolated from a dichlorprop-degrading soil column. It is able to grow on phenoxyalkanoic acid herbicides, such as mecoprop, dichlorprop, 2,4-D, MCPA, and 2,4-DB. Strain MH utilizes both enantiomers of the chiral herbicides mecoprop and dichlorprop as sole carbon and energy sources. Enantiomer-specific uptake systems are responsible for transporting the acidic substrates across the cell membrane. Catabolism is initiated by two enantiomer-specific alpha-ketoglutarate-dependent dioxygenases that catalyze the cleavage of the ether bond of the respective enantiomer to yield the corresponding phenol and pyruvate. Therefore selective degradation of the enantiomers of mecoprop and dichlorprop by strain MH is not only due to enantioselective catabolism but also to enantioselective transport.

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Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(RS)-2-(2,4-dichlorophenoxy)propanoic acid] by Sphingomonas herbicidovorans MH. [J Bacteriol. 1999]

Purification and characterization of two enantioselective alpha-ketoglutarate-dependent dioxygenases, RdpA and SdpA, from Sphingomonas herbicidovorans MH. [Appl Environ Microbiol. 2005]

Complete microbial degradation of both enantiomers of the chiral herbicide mecoprop [(RS)-2-(4-chloro-2-methylphenoxy)propionic acid] in an enantioselective manner by Sphingomonas herbicidovorans sp. nov. [Appl Environ Microbiol. 1995]

Genetic analysis of phenoxyalkanoic acid degradation in Sphingomonas herbicidovorans MH. [Appl Environ Microbiol. 2004]

Involvement of two alpha-ketoglutarate-dependent dioxygenases in enantioselective degradation of (R)- and (S)-mecoprop by Sphingomonas herbicidovorans MH. [J Bacteriol. 1997]

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Plant Molecular Biology 13: 533–540, 1989.
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Expression of a bacterial gene in transgenic tobacco plants confers resistance to the herbicide 2,4-dichlorophenoxyacetic acid

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Received 1 June 1989; accepted 15 June 1989

Key words: 2,4-dichlorophenoxyacetic acid (2,4-D), detoxification, herbicide resistance, transgenic plants

Abstract

Plants resistant to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were produced through the genetic engineering of a novel detoxification pathway into the cells of a species normally sensitive to 2,4-D. We cloned the gene for 2,4-D monooxygenase, the first enzyme in the plasmid-encoded 2,4-D degradative pathway of the bacterium *Alcaligenes eutrophus*, into a cauliflower mosaic virus 35S promoter expression vector and introduced it into tobacco plants by *Agrobacterium*-mediated transformation. Transgenic tobacco plants expressing the highest levels of the monooxygenase enzyme exhibited increased tolerance to 2,4-D in leaf disc and seed germination assays, and young plants survived spraying with levels of herbicide up to eight times the usual field application rate. The introduction of the gene for 2,4-D monooxygenase into broad-leaved crop plants, such as cotton, should eventually allow 2,4-D to be used as an inexpensive post-emergence herbicide on economically important dicot crops.

Introduction

The introduction of novel traits such as herbicide resistance into agronomically important crop plants is one promising aspect of the application of genetic engineering to plant breeding. Herbicide-tolerant transgenic plants have been produced in a variety of ways, including over-expressing a sensitive target gene product or supplementing the sensitive plant cells with mutant, herbicide-resistant copies of the target gene. Detoxification strategies, in which bacterial genes for herbicide detoxification or degradation have been used (e.g. degradative genes for phosphinothricin [3] and bromoxynil [19] detoxification), are an alternate and more straightforward means of creating herbicide-resistant plants since it is unnecessary to first identify the biochemical target of the herbicide. This is particularly important where the effect of the herbicide may be pleiotrophic, as in the case of 2,4-dichlorophenoxyacetic acid (2,4-D), a compound which mimics the action of the plant hormone auxin.

Soil organisms that degrade 2,4-D were identified more than thirty years ago [20] and multi-enzyme pathways for 2,4-D degradation have subsequently been demonstrated in several bacterial genera [4, 8, 22]. In the most intensively studied organism, *Alcaligenes eutrophus*, the 2,4-D degradative pathway comprises six specialized enzymes, encoded by a 75 kb plasmid designated pJP4, which convert 2,4-D to chloromaleylacetic

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acid, a compound subsequently metabolized to succinic acid [5]. The genes for these six enzymes (*tfdA-F*) have been mapped by transposon insertion and the sequences of three of the genes (*tfdA*, *tfdC* and *tfdD*) have been determined [5, 11, 21].

The first gene in the 2,4-D degradation pathway (*tfdA*) encodes a 2,4-D monooxygenase which converts 2,4-D into 2,4-dichlorophenol by cleavage of the aliphatic side chain [21]. Preliminary evidence on the biological effects of 2,4-dichlorophenol suggests that this degradation product is less toxic to plants than the herbicide [18]. Accordingly, we cloned the gene for the 2,4-D monooxygenase (*tfdA*), transferred it into tobacco plants and demonstrated that transgenic plants expressing this enzyme exhibit tolerance to the herbicide 2,4-D.

Materials and methods

Manipulation of the 2,4-D monooxygenase gene (tfdA)

Alcaligenes eutrophus strain JMP134 carrying the 2,4-D degradative plasmid pJP4 was generously provided by R.H. Don (University of Queensland, Australia). Following conjugative transfer to *Escherichia coli* MC1061, plasmid DNA was isolated, cleaved with *Hind* III and cloned into the *Hind* III site of pUC119 by standard techniques [16]. A transformant bearing the 21 kb *Hind* III B fragment of pJP4 cloned in pUC119 then served as the source of DNA for further manipulation of the *tfdA* gene. The *Hind* III fragment was reduced by successive digestion with *Bam* HI, *Sal* I, *Xba* I and *Nco* I to give a 980 bp fragment bounded by *Bam* HI linkers (GGGATCCC, Boehringer Mannheim) at the filled-in *Xba* I and *Nco* I sites (Fig. 1A). Analysis of the *tfdA* sequence [21] had revealed the presence of an out-of-frame ATG codon 95 bp upstream from the GTG translational start codon, and this sequence was removed by BAL-31 digestion [16] of 20 bp from the 5′ end of the subclone. Finally, oligomutagenesis was

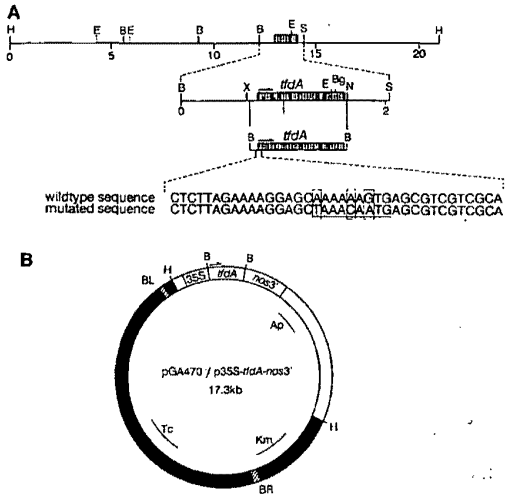


Fig. 1. Cloning of the gene for 2,4-D monooxygenase (*tfdA*) from *Alcaligenes eutrophus* strain JMP134. A. The 960 bp *Bam* HI-*Bam* HI fragment was produced from the 21 kb *Hind* III B fragment of plasmid pJP4 by successive subcloning and BAL-31 digestion as described in Materials and methods. This clone was subjected to oligomutagenesis in the vicinity of the bacterial GTG translational start codon (base changes are boxed) to produce a plant consensus sequence and ATG translational start codon (underlined). The scale beneath each map is in kilobase pairs. Restriction endonuclease sites are: B, *Bam* HI; Bg, *Bgl* II; E, *Eco* RI; H, *Hind* III; N, *Nco* I; S, *Sal* I; X, *Xba* I. The arrow indicates the direction of translation of *tfdA*. B. Schematic representation of the *tfdA* gene cloned between the cauliflower mosaic virus 35S promoter and the nopaline synthase (*nos*) 3′ polyadenylation signal of pJ35SN and inserted into the binary vector plasmid pGA470. Km designates the neomycin phosphotransferase gene which mediates kanamycin resistance in transformed plants; Ap and Tc designate the bacterial genes for ampicillin and tetracycline resistance, respectively. B_L and B_R designate the left and right borders of the T-DNA, respectively.

conducted using an adaptation of an oligonucleotide deletion method [6] to convert the GTG start codon to an ATG and to produce a sequence which matches a published plant consensus for the region adjacent to the translation start [14]. The 37 base oligonucleotide CTCTTAGAA AAGGAGCTAAACA*ATG*AGCGTCGTC GCA was synthesized using an Applied Biosystems 380A DNA synthesizer and correct mutagenesis of the start codon region confirmed by dideoxy sequencing.

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Construction of gene fusions

The *tfdA* gene was excised as a 960 bp *Bam* HI fragment and cloned into the unique *Bam* HI sites of three plant expression vectors based on the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (*nos*) 3′ polyadenylation signal. Plasmid pJ35SN was constructed by replacing the *Hin*d III-*Bam* HI *nos* promoter-chloramphenicol acetyltransferase coding sequences of pNCAT4 (obtained from L. Herrera-Estrella and M. Van Montagu, Rijksuniversiteit, Gent, Belgium), with a *Hin*d III-*Bam* HI fragment containing the 35S promoter of p35SCN [23], and subcloning a *Sca* I-*Hin*d III fragment derived from this construct (comprising 35S promoter and *nos* 3′ separated by *Bam* HI, *Sma* I and *Eco* RI sites) into *Sca* I-*Hin*d III cleaved pUC19. Plasmid p3x35SN was constructed by cloning two additional copies of the 35S promoter upstream enhancer region [15], bounded by *Sal* I and *Eco* RV sites, 5′ to the 35S promoter of pJ35SN. All three enhancer regions were in the same orientation. Plasmid p35SAMVN was constructed by cloning a synthesized 44 base oligonucleotide equivalent to the alfalfa mosaic virus (AMV) leader [10] and bounded by *Bgl* II and *Bam* HI sites into the *Bam* HI site of pJ35SN. The unmodified GTG containing *tfdA* coding sequence was also cloned into pJ35SN in both orientations.

Construction of binary vectors and plant transformation

As illustrated in Fig. 1B, the 35S-*tfdA-nos* constructs were cleaved with *Hin*d III and cloned into the *Hin*d III site of the binary vector plasmid pGA470 [1]. Triparental mating with selection as described [7] was employed to transfer the binary vector constructs from *E. coli* to the avirulent *Agrobacterium tumefaciens* strain LBA 4404 Rif^r [12]. Constructions in *Agrobacterium* were verified by restriction and DNA blotting analysis. Leaves of *Nicotiana tabacum* cv. Wisconsin 38 were transformed by these *Agrobacterium* strains and shoots were regenerated on kanamycin medium essentially as described [7]. Transgenic kanamycin-resistant plants transformed by these promoter constructs were designated TFD 1, 5 or 8 (corresponding to *tfdA* in the coding orientation in pJ35SN, p3x35SN or p35SAMVN, respectively), or TFD 3 (corresponding to *tfdA* in the inverse orientation in pJ35SN). Transgenic plants were maintained under antibiotic selection in aseptic containers and young leaves were periodically harvested for analysis. Selected transgenic plants were transferred to soil in a biosafety containment glasshouse and propagated for seed.

2,4-D monooxygenase assay

Bacterial enzyme extracts were prepared by sonication of cultures suspended in 0.02 M potassium phosphate buffer (pH 7.3), while plant enzyme extracts were prepared by grinding tissue with sand in the presence of 0.02 M potassium phosphate buffer, 2% insoluble polyvinylpyrrolidone and 10 mM dithiothreitol. Extracts were equilibrated for protein content and assayed for 2,4-D monooxygenase activity in the presence of 0.01–0.03 μCi 2,4-dichloro[*ring*-U-^{14}C]phenoxyacetic acid (Amersham; purified by ethyl acetate extraction), 1 mM cysteine and 20 mM NADH. After being flushed with O_2, the assay mixes were incubated with shaking at 30 °C for 1–3 h. The reaction products were extracted into chloroform and separated by thin-layer chromatography (TLC) using benzene-methanol (19 : 1, v/v) as solvents [9]. Phenolic compounds were detected on the chromatogram by staining with diazotized *p*-nitroaniline followed by $NaHCO_3$ [2] or by autoradiography with Fuji X-ray film for 1–14 days.

2,4-D toxicity tests

Tobacco leaf discs of approximately 1 cm in diameter were punched aseptically from fresh leaves and plated on shoot regeneration media [17] containing 0.2, 0.8, 3.2 or 12.8 mg/l 2,4-D

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and incubated for 60 days before shoot and callus tissue was weighed for comparison. Leaf discs from wild-type plants were routinely plated as internal controls on test plates. F_1 seeds generated by self-pollination from wild-type and transgenic plants were surface-sterilized and plated on modified Murashige and Skoog media (no vitamins, 10 g/l sucrose) containing 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 mg/l 2,4-D or 200 mg/l kanamycin and incubated for 14 days before relative germination rates were determined. At 4–8 weeks after germination, seedlings were sprayed until dripping wet with a range of 2,4-D (isopropyl ester in 50% acetone) concentrations (0, 10, 30, 100, 300, 1000 and 3000 mg/l) and were observed for detrimental effects over a period of three weeks.

Results

Cloning and expression of tfdA in bacteria and plants

The 2,4-D monooxygenase gene (*tfdA*) of *A. eutrophus* plasmid pJP4 possesses an 861-nucleotide open reading frame [21]. We cloned in *E. coli* a 960 bp segment comprising the complete *tfdA* gene bounded by 5′ and 3′ non-coding sequences of approximately 90 bp and 10 bp, respectively (Fig. 1A). Oligomutagenesis of three bases in the 5′ region of the gene converted the GTG translational start codon of the native gene to an ATG and produced a plant consensus sequence [TAAACA] immediately upstream of this codon (Fig. 1A).

Both unmodified (GTG) and modified (ATG) *tfdA* genes were fused to the β-galactosidase promoter of pUC plasmids and expression of 2,4-D monooxygenase in *E. coli* was induced by culture in the presence of isopropylthiogalactoside (IPTG). In order to confirm that the cloned and mutated *tfdA* fragment did encode a fully active 2,4-D monooxygenase, bacterial cell extracts were assayed for their ability to convert ^{14}C-labelled 2,4-D into 2,4-dichlorophenol (Materials and methods). An abundant radiolabelled product was detected in extracts from bacteria harbouring either the unmodified or modified *tfdA* gene (Fig. 2, lanes 2 and 3), but was not observed in the enzyme-free control (Fig. 2, lane 5), nor in extracts of *E. coli* containing pUC118 or the modified *tfdA* gene cloned in the inverse orientation (Fig. 2, lanes 1 and 4). This product exhibited a TLC R_F value of approximately 0.46 for the benzene-methanol solvent system and co-migrated with authentic 2,4-dichlorophenol (BDH). Sufficient quantities of the enzymic product were present to demonstrate the characteristic purple colour of 2,4-dichlorophenol by direct staining with diazotized p-nitroaniline.

Assays for 2,4-D monooxygenase activity were conducted on up to thirty different plants produced by transformation with each of the constructs and on the untransformed wild-type tobacco W-38. Unlike the *E. coli* extracts, the extracts from control tobacco plants were able to slowly metabolize 2,4-dichlorophenol by an, as yet, unknown mechanism. This assay was therefore not an accurate measure of the level of 2,4-D monooxygenase present in plant extracts, but did give a qualitative indication of the level of expression. Transgenic plants showing the highest level of 2,4-dichlorophenol were analysed further.

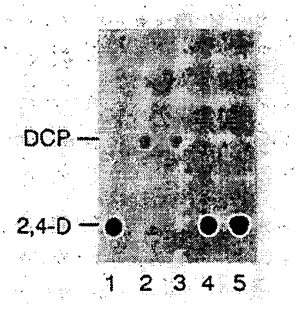


Fig. 2. Chromatogram of 2,4-D monooxygenase assay. Bacterial extracts were assayed for 2,4-D monooxygenase activity and the chromatogram was exposed to X-ray film as described in Materials and methods. Bands due to 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol (DCP) are marked. Lanes are: 1, *E. coli* (pUC118); 2, *E. coli* (unmodified *tfdA* cloned in pUC118); 3, *E. coli* (modified *tfdA* cloned in pUC119); 4, *E. coli* (modified *tfdA* cloned in incorrect orientation in pUC119); 5, enzyme-free control.

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Leaf disc assay for resistance to 2,4-D

The increase in tolerance to 2,4-D *in vivo* was determined by assaying the ability of leaf discs from transgenic plants to regenerate shoots in the presence of the herbicide. Wild-type Wisconsin 38 tobacco leaf discs generated shoots on levels of 2,4-D up to 0.2 mg/l but were inhibited by more than 0.4 mg/l of the herbicide. At these higher levels of 2,4-D, large quantities of undifferentiated callus were produced, presumably as a response to the presence of the auxin analogue. In similar tests conducted with 2,4-dichlorophenol, W-38 tobacco-generated shoots at levels as high as 45–50 mg/l (above which tissue was bleached and killed), implying that 2,4-dichlorophenol is 100-fold less toxic to tobacco than the herbicide 2,4-D when applied externally.

A 2,4-D concentration of 0.8 mg/l, too toxic for the formation of shoots by wild-type tobacco, was used to test for transgenic plants tolerant to high levels of 2,4-D. This level of herbicide prevented the growth of shoots from explants of the non-transformed wild-type, inverse orientation of the ATG construction (TFD 3-1 plants), both orientations of the GTG constructions, together with at least one of the tested transgenic plants expressing low levels of monooxygenase (TFD 8-24). This level of 2,4-D did not adversely affect the formation of shoots by a number of transgenic plants, from all three different promoter constructs containing the ATG modified *tfdA* coding region, that expressed high levels of monooxygenase activity in the *in vitro* assay. Those plants containing the AMV leader region construction appeared marginally better, e.g. TFD 8-1, 8-2, 8-6 and 8-14 (Fig. 3), and these were analysed in most detail. Some of these transgenic plants generated shoots on levels of 2,4-D as high as 12.8 mg/l, representing a 32-fold increase in tolerance to the herbicide compared with the inverse orientation control TFD 3 and wild-type plants. Attempts to select directly for 2,4-D-resistant plants in transformation experiments were, however, unsuccessful.

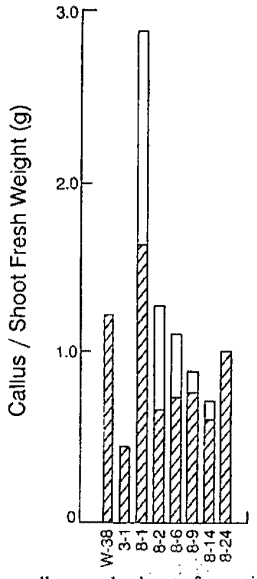


Fig. 3. Tobacco callus and shoot formation on media containing 2,4-D. Tobacco leaf discs were plated on shoot-inducing media containing 0.8 mg/l 2,4-D. Wild-type and transgenic callus (hatched) and shoot (open) formation is expressed as average fresh weight (g).

Seed germination assay for resistance to 2,4-D

Table 1 presents the germination data for seed derived by self-pollination of wild-type and transgenic plants. Germination of wild-type and control TFD 3 seed was affected by concentrations

Table 1. Germination of seed derived from wild-type and transgenic plants on 2,4-D and kanamycin expressed as a percentage of viable seed (50 seeds/treatment).

Plant	2,4-D (mg/l)						Kanamycin 200 mg/l
	0.1	0.2	0.4	0.8	1.6	3.2	
W-38	69	0	0	0	0	0	
TFD 3-1	57	0	0	0	0	0	60
TFD 1-1	85	54	62	52	4	0	80
TFD 5-2	86	41	0	0	0	0	96
TFD 5-10	90	78	68	40	0	0	68
TFD 5-27	89	73	64	73	9	0	80
TFD 8-1	92	67	63	69	33	0	94
TFD 8-6	92	92	81	65	22	10	92
TFD 8-14	80	74	66	50	4	0	60
TFD 8-24	52	10	0	0	0	0	96

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of 2,4-D as low as 0.1 mg/l and was completely inhibited at 0.2 mg/l. In contrast, some transgenic seed was able to germinate on levels of 2,4-D up to 3.2 mg/l, an increase in tolerance to the herbicide of approximately 30-fold.

The seed germination assay was further employed to investigate cross resistance to other phenoxyacetic acid herbicides, including 2 methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T). Seed from TFD 8-6, the most resistant in the plate assays, showed more resistance than wild-type seed to MCPA in the range 0.025–0.1 mg/l, and 20% of the transgenic seed was able to germinate at 0.2 mg/l, a level which prevented germination of the wild type. Transgenic seed was no more tolerant to 2,4,5-T than the wild-type seed.

2,4-D resistance in young plants

Progeny germinated from wild type, TFD 8-1 and TFD 8-6 seed were transferred to soil and grown for 4–8 weeks before being sprayed with concentrations of herbicide ranging from 0–3000 mg/l. Wild-type plants were affected by levels as low as 10 mg/l and were killed by levels of 300 mg/l or more (Fig. 4A). Transgenic plants were much more tolerant to the herbicide, being unaffected by 300 mg/l 2,4-D (Fig. 4A), only slightly affected by 1000 mg/l (data not shown), and even surviving 3000 mg/l 2,4-D (Fig. 4B).

An estimate of the field application rate corresponding to our experimental spraying regime suggests that 1000 mg/l is approximately equivalent to 1–2 kg of 2,4-D per hectare. For weed control, fields are normally sprayed with 0.25–0.5 kg/ha, hence, the transgenic tobacco F_1 progeny can survive spraying with 2,4-D concentrations 4–8 times the normal field application of the herbicide.

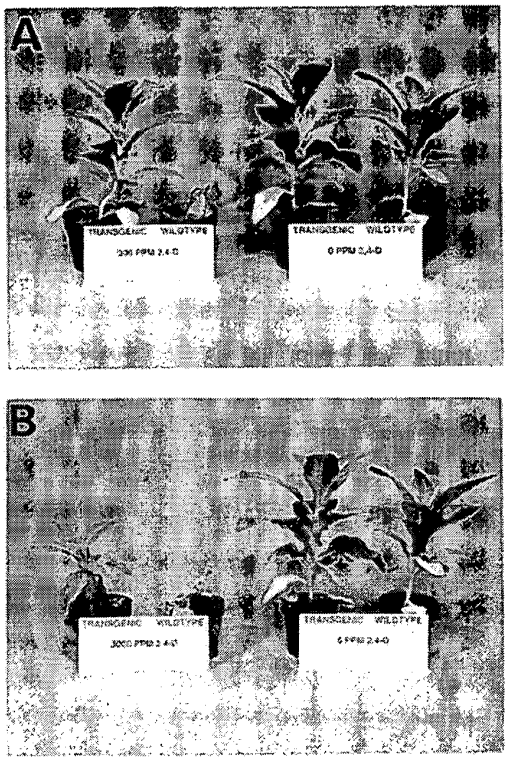


Fig. 4. Spraying of young tobacco plants with 2,4-D. Wild-type (W-38) and transgenic (TFD 8-6) plants were sprayed with (A) 300 mg/l 2,4-D, or (B) 3000 mg/l 2,4-D.

Discussion

The first gene in the 2,4-D degradative pathway of the bacterium *Alcaligenes eutrophus* (*tfdA*) was cloned into three different CaMV 35S promoter-nopaline synthase 3′ expression vectors, and introduced into tobacco plants by *Agrobacterium*-mediated transformation. The bacterial gene had been modified by oligomutagenesis to produce a plant consensus translation start sequence upstream of the translation initiation codon, changed from a GTG to ATG. Many of the transgenic plants demonstrated increased tolerance to 2,4-D as determined by the generation of shoots from leaf discs in the presence of high levels of the herbicide, while plants transformed with an unmodified *tfdA* gene did not exhibit improved tolerance to 2,4-D, indicating that this gene is not expressed efficiently in plants. Resistant plants were produced from all three promoter constructs. One of the most resistant contained the AMV leader region, purported to increase trans-

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lational efficiencies of mRNAs containing this sequence [13]. More intensive studies will be required to fully evaluate the effectiveness of the different promoter constructs in transgenic plants.

A TLC assay for 2,4-D monooxygenase activity in bacterial extracts was not suitable for the accurate determination of enzyme activity in plant extracts, but did enable the initial selection of potential 2,4-D-resistant transgenic plants. A strong correlation was observed between this assay and the three *in vivo* methods (leaf disc, seed germination and spray toxicity) of assessing resistance to 2,4-D. An alternative assay, using antibodies to 2,4-D monooxygenase, is being developed for the early detection of enzyme in transformed tissue from cotton and other plants.

The seed germination assays suggest that high levels of resistance to the herbicide are dependent on both the possession of multiple copies of the *tfdA* gene and the site of integration of the T-DNA in the genome. Seed segregation ratios in the presence of discriminating doses of 2,4-D give some indication of the number of gene insertions in each transgenic plant and support Southern blot analyses of genomic DNA from the parental plants. These results indicate that more than one copy of the *tfdA* gene is present in the most resistant plants such as TFD8-6.

Transgenic seed was found to be slightly resistant to the herbicide MCPA, a result consistent with the observation that *A. eutrophus* can slowly metabolize this compound, which is structurally similar to 2,4-D. The fact that there was no cross-resistance to the closely related herbicide 2,4,5-T implies a high degree of substrate specificity for the 2,4-D monooxygenase. We are currently testing a wide variety of phenoxyacetate herbicides in order to establish the full herbicide resistance spectrum mediated by the 2,4-D monooxygenase and to gain insight into the protein-substrate interactions involved.

Although the level of resistance to 2,4-D in tobacco is adequate, with transgenic progeny able to survive spraying with 2,4-D concentrations some 4–8-fold higher than the usual field application rate, we expect that further improvement in gene expression will be required to obtain adequate protection in other species which are often more sensitive to 2,4-D than tobacco. Despite using a strong promoter linked to the 2,4-D monooxygenase gene, we were unable to detect *tfdA* transcripts by Northern blot hybridization. It may be possible to increase expression by improving transcript stability, perhaps by shortening the non-translated leader sequence of the *tfdA* coding region fragment (currently 90 bp) or substituting new 3′ ends in the expression vectors. These are strategies that have been effective with other gene constructs. The level of resistance in the current transgenic plants will be improved by selecting progeny carrying multiple copies of the *tfdA* gene and sequentially increasing the gene dosage by judicious crossing. This will be important to establish the upper limit of resistance possible with this enzymic detoxification system.

2,4-D is highly active against many species of broad-leaved weeds and is widely employed as a weed control agent on cereal crops. Broad-leaved crop plants, such as cotton, are sensitive to this herbicide, and accidental drift damage from the aerial spraying of neighbouring grain fields can devastate a crop. We are interested in engineering herbicide-resistant cotton that will firstly be resistant to 2,4-D drift damage, and secondly, will enable the post-emergence spraying of cotton with 2,4-D for the control of broad-leaved weeds. The result with the tobacco system suggests that the introduction and expression of the *tfdA* gene in cotton and other broad-leaved crops should provide the necessary level of herbicide tolerance to achieve these objectives.

Acknowledgements

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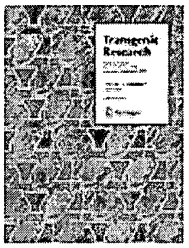
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Journal Article



Cotton plants transformed with a bacterial degradation gene are protected from accidental spray drift damage by the herbicide 2,4-dichlorophenoxyacetic acid

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Abstract The agronomic performance of broad leaved crop plants such as cotton would be greatly improved if genetically-engineered resistance to broadleaf herbicides could both protect the plants from accidental spray drift damage and allow the suppression of problem broadleaf weeds by chemical means. Following *in vitro* modification and the addition of plant expression signals, the gene for 2,4-*D* monooxygenase, a bacterial enzyme that degrades the broadleaf herbicide 2,4-dichlorophenoxyacetic acid (2,4-*D*), was introduced into cotton plants by *Agrobacterium*-mediated transformation. First generation homozygous progeny of regenerated transgenic cotton plants carrying this gene exhibited up to a 50–100 fold increase in tolerance to 2,4-*D* compared with untransformed controls, and glasshouse trials suggest that the genetically-engineered plants would be completely protected from spray drift of 2,4-*D*, at least up to the normal field application rates commonly used on neighbouring cereal crops.

Bayer EX1005-0426 (1 of 2)

Keywords transgenic cotton plants - 2,4-*D* herbicide tolerance - herbicide detoxification

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ORIGINAL PAPER

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Substrate specificity of chlorophenoxyalkanoic acid-degrading bacteria is not dependent upon phylogenetically related *tfdA* gene types

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Abstract The phenoxyalkanoic acid herbicides constitute a group of chemically related molecules that have been widely used for over 50 years. A range of bacteria have been selected from various locations for their ability to degrade these compounds. Previously reported strains able to utilise 2,4-dichlorophenoxyacetic acid (2,4-D) include, *Ralstonia eutropha* JMP134, *Burkholderia* sp. RASC and *Variovorax paradoxus* TV1 and *Sphingomonas* sp. AW5 able to utilise 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). In addition a novel set of mecoprop-degrading strains including *Alcaligenes denitrificans*, *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2 are here described. It has been reported recently that TfdA enzymes, initially reported to have a role in 2,4-D catabolism are also involved in the first-step cleavage of related phenoxyalkanoate herbicides. However, a diversity of *tfdA* gene sequences have been reported. We relate the *tfdA* gene type to the metabolic ability of these strains. The *tfdA*-like genes were investigated by polymerase chain reaction amplification using a set of specific *tfdA* primers. Degradation ability was observed via phenol production from a range of unsubstituted and substituted phenoxyalkanoics including, 2,4-D, 2-methyl 4-chlorophenoxyacetic acid (MCPA), racemic mecoprop, (*R*)-mecoprop, 2-(2,4-dichlorophenoxy) propionic acid (racemic 2,4-DP), 2,4,5-T, 2,4-dichlorophenoxybutyric acid (2,4-DB), 4-chloro-2-methylphenoxybutyric acid (MCPB) and phenoxyacetate. Mecoprop-degrading strains showed partial *tfdA* sequences identical to the one described for *V. paradoxus* TV1 (a strain isolated on 2,4-D). However, substrate specificity was not identical as *V. paradoxus* exhibited greatest activity towards 2,4-D and MCPA only, whereas the mecoprop-degrading strains showed intense activity towards 2,4-D, MCPA, racemic mecoprop and (*R*)-mecoprop as substrates. However, *Sphingomonas* sp. AW5 which has been shown to carry a very different *tfdA*-like gene was the only strain to utilise the phenoxybutyric acid MCPB as a sole carbon source. In this study, we thus demonstrate that sequence diversity is not related to substrate specificity within the *tfdA*-like gene family. However, phylogenetically unrelated sequences may govern substrate specific activity.

Keywords Mecoprop · 2,4-Dichlorophenoxyacetic acid · *tfdA* · Biodegradation · α-Ketoglutarate-dependent dioxygenase

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Introduction

The structurally related herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl 4-chlorophenoxyacetic acid (MCPA), 2-(2-methyl 4-chlorophenoxy) propionic acid (mecoprop), 2-(2,4-dichlorophenoxy) propionic acid (2,4-DP), 2,4-dichlorophenoxybutyric acid (2,4-DB), 4-chloro-2-methylphenoxybutyric acid (MCPB) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have been widely applied to cereal crops to control dicotyledonous weeds worldwide. The microbial metabolism of 2,4-D is well understood and is a paradigm for the degradation of other chloroaromatic compounds (Häggblom 1992). The first 2,4-D degradation pathway was established in an *Arthrobacter* species and subsequently demonstrated in numerous 2,4-D-degrading strains (Bollag et al. 1968; Evans et al. 1971; Gaunt and Evans 1971).

The catabolism of 2,4-D by *Ralstonia eutropha* JMP134 (formerly *Alcaligenes eutrophus* JMP134) involves a number of steps including side chain removal, hydroxylation of the resulting 2,4-dichlorophenol (2,4-DCP), opening of the 3,5-dichlorocatechol ring and then conversion of 2,4-dichloro-*cis*,*cis*-muconate to succinate for cell metabolism. In *R. eutropha* JMP134, these activities are encoded by the 2,4-D degradation genes (*tfdA-F*) located on the well-characterised plasmid pJP4

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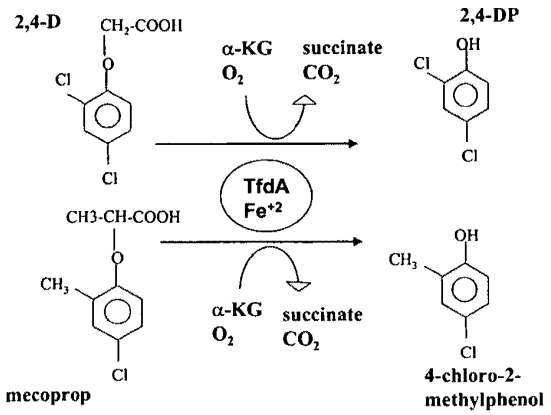


Fig. 1 The catabolic activity of the TfdA enzyme in 2,4-Dichlorophenoxyacetic (*2,4-D*) and mecoprop degradation. *KG* α-Ketoglutarate, *2,4-DCP* 2,4-dichlorophenol

(Don and Pemberton 1981; Don et al. 1985). The *tfdA* gene that encodes the first step of the pathway (i.e. the removal of the side chain of the 2,4-D molecule) is a key feature both functionally and evolutionarily. Fukumori and Hausinger (1993) suggested that the *tfdA* gene may have evolved from a parent gene involved in the metabolism of taurine (*tauD*) (Van der Ploeg et al. 1996).

The mecoprop degradation pathway has been shown to be convergent with the 2,4-D pathway (Tett et al. 1997) and *tfd*-like genes have been shown to be found in mecoprop degraders (Smejkal et al. 2000a). This has also been reported for the metabolism of 2,4-DP (Saari et al. 1999). However, on the basis of the reaction sequence for 2,4-D degradation, the first metabolite detected in mecoprop metabolism by *Alcaligenes denitrificans* is 4-chloro-2-methylphenol (MCP) as opposed to 2,4-DCP (Fig. 1). This activity may be facilitated by an enzyme characteristic of an α-ketoglutarate-dependent dioxygenase (TfdA) (Tett et al. 1997).

It is known that an α-ketoglutarate-dependent dioxygenase from *R. eutropha* JMP134 can utilise MCPA in addition to 2,4-D as a substrate. It also utilises the unsubstituted phenoxyacetic acid, although with a weak induction of the enzyme (Pieper et al. 1988). Many 2,4-D-degrading strains do not have wide substrate specificity for the chlorophenoxyalkanoic herbicides and are often limited to 2,4-D as a growth substrate (Ka et al. 1994; Hoffman et al. 1996). Conversely, mecoprop-degrading strains are frequently able to degrade alternative chlorophenoxyalkanoic acids such as 2,4-D, MCPA and 2,4-DP (Tett et al. 1994; Zipper et al. 1996; Müller et al. 1999; Marriott et al. 2000). However, their ability to utilise a range of phenoxyalkanoates has not previously been described.

Recently *tfdA*-specific PCR primers were developed (Vallaeys et al. 1996), allowing specific amplification of different families of the *tfdA* gene within many 2,4-D-degrading strains. Studies have shown that there are four, diverse types of *tfdA* genes among 2,4-D-degrading bacteria (McGowan et al. 1998; Vallaeys et al. 1996, 1999). The *tfdA* gene type was also found to be incongruent from the species type, indicating that this gene must have originated as a result of gene transfer between bacterial species (McGowan et al. 1998). Likewise, the *tfdA* gene has been found to be incongruent with analyses of the *tfdB* and *tfdC* genes (Vallaeys et al. 1999). The functional role of *tfdA* gene sequence diversity and its overall activity in nature is not yet fully understood. Therefore the question arises; is there a link between the *tfdA* gene type and particular substrate specificity towards a range of related substrates?

Here we present the phylogenetic relationship of *tfdA*-like genes in mecoprop-degrading strains to the four types of *tfdA* gene found in 2,4-D-degrading bacteria. Subsequent determination of gene sequence and type will permit comparisons of *tfdA* gene type and substrate specificity of its encoded α-ketoglutarate-dependent dioxygenase to different chlorophenoxyalkanoic substrates. We provide evidence that there is no apparent phylogenetic functional relationship between substrate specificity and *tfdA* gene type. Conversely, substrate utilisation range may be governed by phylogenetically distinct genes that function in the same way as the *tfdA* gene. Equally this specialisation may be due to a permease gene which encodes an enzyme involved in substrate-specific uptake, or even enzyme activities at subsequent steps of the degradation pathway as encoded by the *tfdB-tfdF* genes.

Materials and methods

Chemicals

Mecoprop, (*R*)-mecoprop, 2,4-DP, MCPA, 2,4-D, MCPB and 2,4-DB were generously donated by A. H. Marks (Bradford, UK). 2,4,5-T was obtained from Sigma (UK), phenoxyacetic acid from Merck, and 4-nitrophenoxyacetic acid from T.C.I. (Tokyo).

Bacterial strains and culture conditions

Bacterial strains are listed in Table 1. The strains were routinely grown at 30°C in 100-ml batch cultures containing (g l^{-1} distilled water): $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; Na_2EDTA, 0.12; NaOH, 0.02; $ZnSO_4$, 0.004; $CuSO_4.5H_2O$, 0.001; Na_2SO_4, 0.0001; Na_2MoO_4, 0.001; $CoCl_2$, 0.0001; $MnSO_4$, 0.0004; and 0.5 ml concentrated H_2SO_4. The pH of the medium was adjusted to 7.0 and the respective isolation substrate was added at a concentration of 3 mM (2,4-D, mecoprop, 2,4,5-T).

Partial sequencing of the *tfdA*-like gene and phylogenetic analyses

Plasmid DNA was extracted from the mecoprop-degrading strains (Wheatcroft and Williams 1981) then amplified with specific primer sets for the *tfdA* gene as described by Vallaeys et al. (1996). Aliquots of 5–10 µl of the PCR solution were analysed on horizontal 1% agarose gels (BDH) in TBE buffer (40 mM TRIS-HCl, 4 mM sodium acetate, 1 mM EDTA, pH 7.9) using the Biorad DNA sub cell. Electrophoresis was carried out for 30 min at 60 V. Ethidium bromide-stained gels were then photographed on an Alpha Innotech Corporation UV transilluminator connected to Chemilmager 4000i v4.04. Sequencing reactions were performed by MWG Bio-

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Table 1 Bacterial strains used in this study

Strain		Plasmid	Isolated on	Origin	*tfdA* gene type[b]	*tfdA* Accession number/reference	Reference
Ralstonia eutropha	JMP134	pJP4 (85 kb)	2,4-D	Australia	1	M16730	Don and Pemberton (1981)
Variovorax paradoxus	TV1	pTV1	2,4-D	France	4	U65531	Vallaeys et al. (1998)
Burkholderia sp.	RASC	–	2,4-D	USA	2	U25717	Fulthorpe et al. (1995)
Sphingomonas sp.	AW5	pAW5 (50 kb)[a]	2,4,5-T	UK	ND	–	Weightman (unpublished data)
Alcaligenes denitrificans		pCS3 (approx. 225 kb)	Mecoprop	UK	4	Saari et al. (1999)	Tett et al (1994)
Alcaligenes sp.	CS1	pCS1 (approx. 225 kb)	Mecoprop	UK	4	AF181982	(This study)
Ralstonia sp.	CS2	pCS2 (approx. 225 kb)	Mecoprop	UK	4	AF182758	(This study)
Escherichia coli		pUC19::*tfdA*	–	–	1	–	Holben et al. (1995)

[a] Location of degradation genes on this plasmid have not yet been determined

[b] The tfdA gene type is as defined in Vallaeys et al. (1996)
ND not determined

tech (Ebersberg, Germany). The forward and reverse *tfdA* primer consensus sequences were submitted to Genbank under accession numbers AF181982 (*Alcaligenes* sp. CS1) and AF182758 (*Ralstonia* sp. CS2) (Smejkal et al. 2000b, 2000c). Fasta and Blast subroutines (Pearson and Lipman 1988) were used to search for related sequences in the GenBank, EMBL, and RDP databases. The CLUSTAL W program (Thompson et al. 1994) was used for multiple sequence alignments. The PHYLIP package (Felsenstein 1989) was used for distance estimation, using the Kimura (Kimura 1980) method, and dendogram construction, using neighbor-joining (Saitou and Nei 1987). Bootstrap values were estimated according to Zharkikh and Li (1992) with 100 replicates.

The following organisms were included in the phylogenetic analysis (accession numbers in parentheses): *β-proteobacterium* B6–9 (U43196), *Rhodoferax* sp. P230 (AF176240), *Achromobacter xylosoxidans* (pEST4011) (U32188), *Variovorax paradoxus* (pTV1) (U65531), *Halomonas* sp I-18 (U22499), *Burkholderia* sp. RASC (U25717), *Burkholderia* sp. TFD39 (U43197), *Ralstonia eutropha* JMP134 (M16730), *β-proteobacterium* TFD23 (U43276) and *Escherichia coli* K-12 (U00096).

Use of 4-nitrophenoxyacetic acid to detect TfdA activity

The production of a bright yellow 4-nitrophenol chromophore was used to detect whole-cell TfdA activities in the strains described in Table 1, when grown on minimal medium plates containing a range of substituted and unsubstituted phenoxy compounds. Colonies were wetted with a freshly prepared assay solution containing 10 mM-4-NPAA, 50 μM $Fe(NH_4)_2(SO_4)_2$, and 50 μM ascorbate in 20 mM TRIS-HCl; pH 7.4, allowed to dry and then rewetted with an atomiser (Sassanella et al. 1997). Plates were incubated at 30°C with observation at 5 min to 6 h. Control nutrient agar (Oxoid, UK) plates and minimal agar plates containing only 3 mM sodium acetate were employed.

Colorimetric assay spray technique for detection of derivatised phenols

This method relies on the condensation of 4-aminoautipyrine and liberated phenol in the presence of alkaline potassium ferricyanide to produce a red autipyrine dye (AAPPC) and is known as the Emerson reaction (King et al. 1991).

Pre-grown cultures of the strains shown in Table 1 were grown in 10 ml of minimal salts medium containing their respective isolation substrate (2,4-D, Mecoprop, 2,4,5-T) at concentrations of 3 mM. Late log-phase samples were serial diluted in 1/4 strength Ringers solution (Oxoid, UK), the 10^{-5} dilution of each strain was plated onto separate minimal selective agar plates of the following composition (g l^{-1} distilled water): $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; Na_2EDTA, 0.12; NaOH, 0.02; $ZnSO_4$, 0.004; $CuSO_4$, 0.001; Na_2SO_4, 0.0001; Na_2MoO_4, 0.001; $CoCl_2$, 0.0001; $MnSO_4$, 0.0004; agar, 15.0; and 0.5 ml of concentrated H_2SO_4. The pH of the medium was adjusted to 7.0 and substituted and unsubstituted phenoxyalkanoate substrates were added at concentrations of 3 mM. Plates were incubated at 30°C for 24 h, except for *V. paradoxus*, which required 5 days due to a slower growth rate. The colonies were wetted with an 8% potassium ferricyanide solution (in distilled water). The plates were allowed to air dry before being sprayed with a solution of pH-10.0 buffer solution (3.09 g boric acid, 3.73 g potassium chloride, 44 ml of 1 N sodium hydroxide and made up to 1 l with distilled water), 2% 4-aminoantipyrine (in distilled water) and 8% potassium ferricyanide (5:1:1, v/v/v) (King et al. 1991). Triplicate plates were observed for each substrate. Nutrient agar (Oxoid, UK) plates and minimal agar plates containing only 3 mM sodium acetate were used as negative controls. All plates were observed after 10 min incubation at room temperature.

Results

Partial sequencing of the *tfdA*-like gene and phylogenetic analyses

PCR amplification of the plasmid DNA from *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2 using the *tfdA* primer set, gave rise to fragments of approximately 300 bp (Fig. 2). Partial nucleotide sequences were undertaken for these strains and these were compared to existing *tfdA* sequences in Genbank, EMBL, and RDP using Fasta and Blast subroutines. Sequence comparison showed that CS1 and CS2 have *tfdA*-like sequences with

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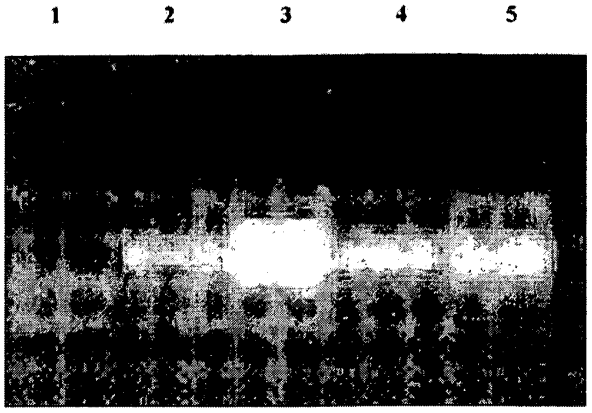


Fig. 2 Polymerase chain reaction amplification of the approximately 300-bp *tfdA* gene fragments from mecoprop-degrading strains. *Variovorax paradoxus* TV1 and *Sphingomonas* sp. AW5 were used as positive and negative controls. *Lane 1* pAW5, *lane 2* pCS1, *lane 3* pCS2, *lane 4* pCS3, *lane 5* pTV1

100% similarity to the 2,4-D-degrading *V. paradoxus* TV1, which has a type 4 *tfdA* gene, and to the mecoprop-degrading *A. denitrificans*. The dendogram produced by neighbor-joining shows the phylogenetic relationship between the *tfdA* gene with 2,4-D-degrading strains and the *tfdA*-like gene found in mecoprop-degrading strains (Fig. 3). The sequences showed 77% similarity to the canonical *tfdA* gene from *R. eutropha* JMP134 (type 1 *tfdA*), 93% to *Burkholderia* sp. RASC (type 2 *tfdA*) and 99% to *Halomonas* sp. I-18 (type 4 *tfdA*). No *tfdA* amplification was achieved with the 2,4,5-T-degrading *Sphingomonas* sp. AW5.

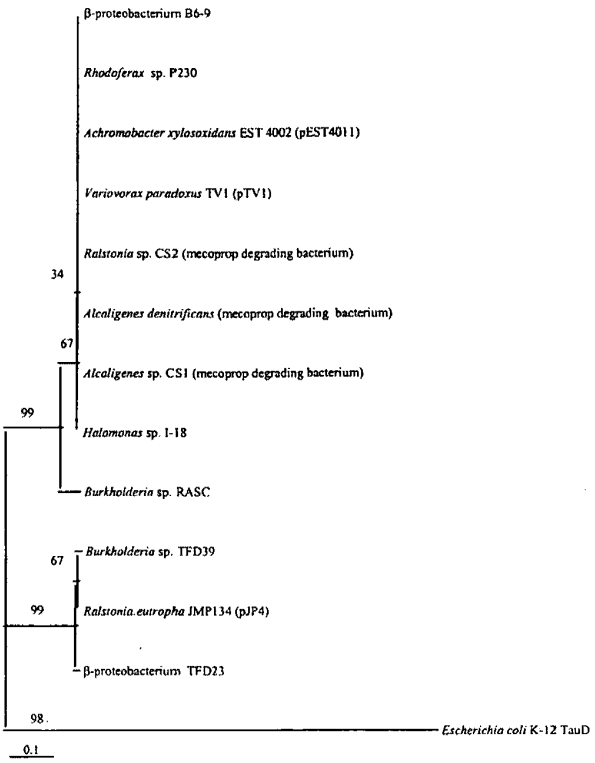


Fig. 3 Neighbor-joining dendogram (Saitou and Nei 1987) of *tfdA* genes from 2,4-D- and mecoprop-degrading bacteria. Bootstrap confidence limits (percentages) are indicated above each branch. A reference outgroup is signified by the taurine (*tauD*)-degrading *Escherichia coli* K-12 (Van der Ploeg et al. 1996). *Scale bar* represents a Jukes-Cantor distance of 0.1

Use of 4-nitrophenoxyacetic acid to detect TfdA activity

All strains studied showed potential TfdA activity as indicated by production of an intense yellow 4-nitrophenol chromophore after 5–10 min on all substrate types tested except control media, thus demonstrating all strains could actively utilise 4-nitrophenoxyacetic acid.

Colorimetric assay spray technique for detection of derivatised phenols

The 2,4-D strains studied exhibited similar specificity for the substrates 2,4-D and MCPA with intense AAPPC production being observed (Table 2). Weak AAPPC was

Table 2 AAPPC production from a set of substituted and unsubstituted phenoxy compounds using the method of King et al. (1991). Observations were performed with all strains pre-grown on their respective isolation media [2,4-dichlorophenoxyacetic acid (*2,4-D*); 2,4,5-trichlorophenoxyacelic acid (*2,4,5-T*); mecoprop]. *MCPA* 2-Methyl 4-chlorophenoxyacetic acid; *2,4-DP* 2-(2,4-dichlorophenoxy)propionic acid; *2,4-DB* 2,4-dichlorophenoxybutyric acid; *MCPB* 2-methyl 4-chlorophenoxybutyric acid; *PA* phenoxyacetate; + intense phenol production on the compounds; +/– weak phenol production; – no phenol production

Strains	Compounds										
	2,4-D	MCPA	Racemic mecoprop	(R)-mecoprop	Racemic 2,4-DP	2,4,5-T	2,4-DB	MCPB	PA	Sodium acetate	Nutrient media
R. eutropha JMP134	+	+	+/–	–	+/–	+/–	–	–	–	–	–
V. paradoxus TVI	+	+	+/–	–	+/–	–	–	–	+	–	–
Burkholderia sp. RASC	+	+	+/–	–	+/–	–	–	–	+	–	–
Sphingomonas sp. AW5	+/–	–	+	–	–	+	–	+	+	–	–
A. denitrificans	+	+	+	+	+/–	–	–	–	–	–	–
Alcaligenes sp. CSI	+	+	+	+	+	+/–	+/–	+/–	–	–	–
Ralstonia sp. CS2	+	+	+	+	+	–	–	–	+	–	–
E. coli	+	+	–	–	–	–	–	–	+	–	–

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produced after these strains were grown on racemic mecoprop and 2,4-DP but not the (*R*)-mecoprop enantiomer. Additionally *R. eutropha* JMP134 expressed weak TfdA activity towards 2,4,5-T but no activity towards phenoxyacetate as substrates. Conversely, intense AAPPC was produced when *Sphingomonas* sp. AW5 was grown on racemic mecoprop, 2,4,5-T, MCPB and phenoxyacetate as growth substrates (Table 2).

Mecoprop-degrading strains, *A. denitrificans*, CS1 and CS2 displayed intense AAPPC production when grown on 2,4-D, MCPA, racemic mecoprop and (*R*)-mecoprop as substrates (Table 2). Intense AAPPC was produced with CS1 and CS2 on racemic 2,4-DP as a substrate whereas there was only weak AAPPC produced for *A. denitrificans* on racemic 2,4-DP (Table 2). Weak AAPPC production was observed for CS1 when 2,4,5-T, 2,4-DB and MCPB were used as substrates (Table 2). Of the mecoprop-degrading strains only CS2 expressed TfdA activity towards phenoxyacetate. No AAPPC was produced on sodium acetate and nutrient media.

Discussion

In many species of bacteria which degrade 2,4-D, methods including *tfdA* gene disruption using transposon mutagenesis (Streber et al. 1987; Suwa et al. 1996; Vallaeys et al. 1998) have shown that divergent *tfdA* genes can play a similar or identical role in the 2,4-D catabolic pathway. *tfdA* gene diversity is not yet understood. Moreover, chlorophenoxyalkanoic acid herbicides were not introduced until after World War II, which is very recent in terms of evolution. This gene diversity is in marked contrast to the degradation of other xenobiotic herbicides, such as atrazine, where there is only one type of *atzA* gene from geographically diverse atrazine-degrading bacteria (De Souza et al. 1998).

Although the role of the *tfdA*-like gene has not been demonstrated in the mecoprop and 2,4-DP degradation pathways, many convergent results suggest that it is indeed involved (Nickel et al. 1997; Saari et al. 1999; Smejkal et al. 2000a). Tett et al. (1997) reported that the enzyme involved in the first step of mecoprop degradation in *Alcaligenes denitrificans* is iron dependent and this is in accordance with the study by Fukumori and Hausinger (1993) suggesting that the first step of mecoprop degradation involves a TfdA. More recently, Saari et al. (1999) showed that in 2,4,-DP catabolism, the first enzyme produced by *A. denitrificans* was characteristic of TfdA because it was α-ketoglutarate and ferrous iron dependent. All of these reports suggest that the first step of mecoprop degradation involves a TfdA enzyme. However, it is necessary to further demonstrate the role of TfdA in both the mecoprop and 2,4-DP degradation pathways by protein sequencing and transposon- or site-directed mutagenesis; this is currently underway.

It has been reported that there are four families of *tfdA* genes in 2,4-D-degrading strains (Vallaeys et al. 1996; McGowan et al. 1998); however, all of the mecoprop strains used in this study have type 4 *tfdA* genes with 100% sequence similarity to *V. paradoxus* TV1 (isolated on 2,4-D). It would therefore be expected that these strains should exhibit identical substrate specificity; however, we report that they all have different specificities that are not influenced by the *tfdA* gene type. Among 2,4-D-degrading strains with distinctive *tfdA* gene types, similar substrate specificity was observed. For example, *R. eutropha* JMP134 (type 1 *tfdA*) (Streber et al. 1987), *Burkolderia* sp. RASC (type 2), chromosomally located *tfdA* gene (Matheson et al. 1996) and *V. paradoxus* TV1 (Vallaeys et al. 1998) all showed greatest activity towards 2,4-D and MCPA with weak activity towards racemic mecoprop and 2,4-DP but no activity towards the (*R*)-enantiomers. These weak activities towards the racemic compounds may be as a result of these strains producing a TfdA which can utilise only one of these enantiomers. This has been demonstrated previously where it was reported that *R. eutropha* JMP134 and *Burkholderia* sp. RASC do have specific activity for the (*S*)-enantiomers of these compounds (Nickel et al. 1997; Saari et al. 1999).

Sphingomonas sp. AW5, isolated on 2,4,5-T, has been reported not to express the *tfdA* gene using *tfdA* primer sets (Vallaeys et al. 1997). However, weak TfdA-like activity was detected with 2,4-D as a substrate. Moreover, intense AAPPC was produced when this strain was grown on 2,4,5-T, racemic mecoprop, MCPB and phenoxyacetate. This may be indicative of trichlorophenoxyacetic acid oxygenase (TftA) activity in the first-step cleavage of these compounds. This enzyme is encoded by the *tftA* gene in the 2,4,5-T-degrading *Burkholderia cepacia* AC1100 (Danganan et al. 1995) and expresses similar properties to TfdA. To date this is the first reported strain with the ability to utilise MCPB as a sole carbon source. In addition, *tfdB*-like and *tfdC*-like genes related to the *tfdB* and *tfdC* genes from 2,4-D-degrading strains have been amplified from AW5 (Vallaeys et al. 1997), suggesting that independent gene recruitment may have occurred.

Although there are several types of *tfdA* gene, it is apparent that substrate specificity must be governed by other unrelated factors. The *tfdB* gene which has a similar sequence to the phenol hydroxylase gene *PheA* (Nurk et al. 1991) may be involved in substrate specificity because there are several types of *tfdB* gene amongst 2,4-D-degrading strains (Vallaeys et al. 1999). The down-stream *tfdC* gene encodes a relaxed substrate chlorocatechol 1,2-dioxygenase and possesses significant sequence similarity to *clcA* (Frantz and Chakrabarty 1987) and the *tcbC* gene (Van der Meer et al. 1991). Further studies are needed to determine any link between the *tfdC* gene and substrate specificity.

A recent study by Leveau et al. (1998) has demonstrated that a gene designated *tfdK* is involved in the uptake of 2,4-D by *R. eutropha* JMP134. This encodes a protein belonging to the major facilitator superfamily (MFS) group of transporter proteins and is related to pro-

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teins responsible for the transport of carboxylated aromatic substances. It could be that the TfdK protein in *R. eutropha* JMP134 is specific to uptake of 2,4-D and MCPA only. This could also be true for the other strains where they have transport proteins exhibiting specificity to particular compounds.

Sequence divergence of the related *tfdA* gene family is not associated with the specific degradation of particular phenoxyalkanoic acid herbicides by the strains studied. It is likely, therefore, that *tfdA* gene diversity pre-existed the post-war "pesticide era" and is not linked to the introduction of different types of related compounds.

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Transgenic Tobacco Plants Expressing a Bacterial Detoxifying Enzyme are Resistant to 2,4-D

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We have used a gene from the soil bacterium *Alcaligenes eutrophus* JMP134 to introduce a herbicide degrading mechanism into plants. The gene, *tfdA*, which encodes a 2,4-dichlorophenoxyacetate monooxygenase (DPAM) catalyzing the first step in the bacterial 2,4-D degradative pathway, has recently been cloned and sequenced. The coding sequence, fused with plant expression sequences was introduced into plants via *Agrobacterium*-mediated gene transfer. The chimeric gene was expressed in tobacco (*Nicotiana tabacum*) under the control of either a constitutive or a light-inducible promoter. Transgenic tobacco lines tolerated elevated levels of 2,4-D in tissue culture, and regenerated plants showed resistance when sprayed with the herbicide.

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Analysis, Cloning, and High-Level Expression of 2,4-Dichlorophenoxyacetate Monooxygenase Gene *tfdA* of *Alcaligenes eutrophus* JMP134

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Plasmid pJP4 of *Alcaligenes eutrophus* JMP134 contains all genes for the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). Five of these genes, *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF*, have recently been localized and cloned (R. H. Don, A. J. Weightman, H.-J. Knackmuss, and K. N. Timmis, J. Bacteriol. 161:85–90, 1985). Gene *tfdA*, which codes for the 2,4-D monooxygenase, has now been found by mutagenesis with transposon Tn*5*. A 3-kilobase fragment of pJP4 cloned in a broad-host-range vector could complement the 2,4-D-negative phenotype of two mutants which lacked 2,4-D monooxygenase activity. The cloned *tfdA* gene was also transferred to *A. eutrophus* JMP222, which is a cured derivative of JMP134. The recombinant strain could utilize phenoxyacetic acid as a sole source of carbon and energy. *Pseudomonas* sp. strain B13, containing the cloned *tfdA*, was able to degrade phenoxyacetic acid and 4-chlorophenoxyacetic acid. Gene *tfdA* was subcloned and analyzed by deletions. Expression of 2,4-D monooxygenase in *Escherichia coli* containing a 1.4-kilobase subfragment was demonstrated by radioisotopic enzyme assay, and a protein of 32,000-dalton molecular mass was detected by labeling experiments. A 2-kilobase subfragment containing *tfdA* has been sequenced. Sequence analysis revealed an open reading frame of 861 bases which was identified as the coding region of *tfdA* by insertion mutagenesis.

Bacteria which degrade halogenated aromatic compounds are of general importance for the detoxification of pesticides and herbicides in nature (17). The extensive use of chlorinated phenoxyalcanoic acid herbicides in agriculture has induced the rapid evolution and dissemination of specific degradative pathways for these compounds in soil and water bacteria. 2,4-Dichlorophenoxyacetic acid (2,4-D) especially is known to be metabolized by organisms belonging to a variety of different bacterial genera, such as *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, and *Pseudomonas* (4, 5, 7, 11–13, 16, 33, 35–37).

Alcaligenes eutrophus JMP134 is one of the best-characterized organisms among this group. It harbors a conjugative plasmid, pJP4, of about 80 kilobase (kb) size, which carries genes essential for the degradation of 2,4-D, 2-methyl-4-chlorophenoxyacetic acid, and 3-chlorobenzoic acid (3-CB) (7). Recently this plasmid has been isolated and characterized physically (8). Five genes involved in the degradation of 2,4-D and 3-CB have already been localized by transposon mutagenesis and cloned in *Escherichia coli*. Functions have been assigned to four of them by biochemical studies (9): *tfdB*, *tfdC*, *tfdD*, and *tfdE* encode 2,4-dichlorophenol hydroxylase, dichlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and chlorodienelactone hydrolase, respectively. Transposon insertion mutation of gene *tfdA* encoding the first enzyme in 2,4-D degradation, which is generally designated as a monooxygenase (9, 35), could not be found in this experiment. Another research group, however, reported the expression of the ability to remove the acetate side chain from 2,4-D by an *E. coli* strain containing a cloned 21-kb *Hin*dIII fragment from pJP4 (1). Characterization of gene *tfdA* would complete our knowledge about the degradative functions in *A. eutrophus* JMP134 and would make it available for genetic engineering studies involving manipulation of microbial organisms to broaden their degrading capacity, or involving the potential use of *tfdA* as a gene specifying herbicide resistance for plants.

We have now isolated transposon mutants of *A. eutrophus* JMP134 inactivated in 2,4-D monooxygenase and have localized gene *tfdA* by cloning and deletion analysis. We have shown the expression of the cloned 2,4-D monooxygenase in different strains, detected the corresponding protein by specific labeling, and determined the nucleotide sequence of a 2-kb fragment containing the *tfdA* coding region.

MATERIALS AND METHODS

Strains and plasmids. *A. eutrophus* JMP134 (7) contains plasmid pJP4 and degrades 2,4-D (Tfd^+) and 3-CB ($3cb^+$). Strain JMP222 is a streptomycin-resistant, pJP4-negative derivative of strain JMP134 and is Tfd^- and $3cb^-$ (7). *Pseudomonas* sp. strain B13 (10) degrades 3-CB and 4-chlorophenol (29). *E. coli* LE392 (27) was used for cloning experiments. *E. coli* HB101 (6) was the recipient for the conjugative transfer of mutated pJP4 derivatives. *E. coli* S17-1 (32) contains a chromosomally integrated RP4 derivative which is able to mobilize broad-host-range plasmids. Strain K38 (=C600)(2) containing plasmid pGP1-2 (34) can express T7 RNA polymerase controlled by a heat-sensitive lambda repressor, and plasmids pT7-5 and pT7-6 (S. Tabor, unpublished data) carry the corresponding T7 promotor in front of a multiple cloning site. Plasmids pVK101 (22), pGSS33 (30) and pKT231 (3) are mobilizable broad-host-range vectors. pSUP2021 is a mobilizable plasmid carrying transposon Tn*5* (specifies kanamycin resistance). For sequencing we used M13tg130 and M13tg131 (21). pDOC37 (D.

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O'Connor, unpublished data) was the source of the omega fragment (15).

Media and cultural conditions. *A. eutrophus* strains were grown with aeration at 30°C. PYE (7) was used as complete medium; liquid PYE medium was supplemented with fructose (10 mM) when cells were grown for preparative plasmid isolation. Minimal medium contained (per liter): K_2HPO_4, 1.6 g; KH_2PO_4, 0.4 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; and $FeSO_4 \cdot 7H_2O$, 0.01 g. Carbon sources were added to the following final concentrations: 2,4-D, 1 mM; 3-CB, 2 mM; 4-chlorophenoxyacetic acid, 1 mM; phenoxyacetic acid (PAA), 4 mM; fructose, 10 mM; and sodium pyruvate, 15 mM. Matings on solid medium were done on PYE agar plates (7). *E. coli* strains were grown in LB medium (25) at 37°C if not otherwise specified. Antibiotics were incorporated at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; streptomycin, 25 μg/ml; and spectinomycin, 80 μg/ml.

Conjugative crosses. For transposon mutagenesis, donor and recipient cells were grown in liquid culture to the late exponential phase and mixed in a 1:1 ratio relative to their optical density. Samples of 1.5 ml of the mixture were spread onto PYE plates and incubated overnight at 30°C. Plate surfaces were then washed with 0.7% NaCl solution, the cell suspension was diluted, and samples were spread onto selective plates.

For complementation tests, mutant strains were grown overnight in liquid culture and spread onto 2,4-D minimal plates, and donor strains were streaked onto plate surfaces. Conjugation and selection were simultaneously done by incubation at 30°C for 14 days.

For all other conjugations, cells were grown in liquid culture overnight, mixed, and spotted onto PYE plates. After incubation at 30°C for 4 to 6 h, cells were suspended in 0.7% NaCl solution, diluted, and spread onto selective plates.

Transposon mutagenesis. The mobilizable transposon carrier plasmid pSUP2021 (32) was transferred by conjugation from the mobilizing donor strain *E. coli* S17-1 (32) to *A. eutrophus* JMP134. Transconjugants were selected from mating mixture on fructose minimal medium containing 380 μg of kanamycin per ml and subsequently screened for loss of 2,4-D degradative ability by replica plating on minimal medium containing 2,4-D as the single carbon source. The absence of degradative functions was also verified by testing on 2,4-D indicator medium (24).

Oxygen uptake assays. *A. eutrophus* wild-type or mutant strains were grown overnight in 250 ml of minimal medium containing 15 mM sodium pyruvate. For induction of 2,4-D-degradative genes, the same medium was used with addition of 3-CB to a final concentration of 1 mM (9). Cells were harvested by centrifugation at 4°C, washed three times in cold minimal medium without carbon source, and finally suspended in a portion of minimal medium to an optical density at 420 nm (A_{420}) of 30.

For assay of 2,4-D monooxygenase or 2,4-dichlorophenol hydroxylase activity, 1 volume of cell suspension was mixed with 9 volumes of minimal medium saturated with oxygen to yield an A_{420} of 3, and background level of oxygen uptake was determined for 10 min. Then substrate was added to a final concentration of 1 mM 2,4-D, 1 mM PAA, or 0.2 mM 2,4-dichlorophenol, and linear decrease of oxygen concentration was measured over 20 min.

Preparative plasmid isolation. Plasmid pJP4 and transposon Tn*5* containing derivatives of pJP4 were isolated from *A. eutrophus* by the procedure of Hansen and Olsen (18). Plasmids from *E. coli* were prepared by the alkaline lysis method described by Maniatis et al. (25).

Radioisotopic 2,4-D assay. For the assay of 2,4-D monooxygenase in *E. coli*, cells of strain K38 containing both pGP1-2 and the pT7 recombinant plasmid were induced for overproduction of enzyme. They were grown at 30°C in 20 ml of LB medium under ampicillin and kanamycin selection. At an A_{590} of 1.0 the temperature was raised to 42°C for 25 min, rifampin was added to a final concentration of 100 μg/ml, and cells were shaken for 2 h at 37°C. Harvesting, washing, and incubation followed the protocol given by Amy et al. (1) with the following modifications: 20 ml of cell suspension was incubated with 0.1 μCi of ^{14}C-labeled 2,4-D in a 250-ml Warburg flask, which was closed with a tapered glass stopper and a serum stopper. A 0.5-ml volume of beta-phenylethylamine was placed in the central vial, and 2 ml of 1 N H_2SO_4 was injected into the surrounding cell suspension. After 1 h of gentle shaking, the trapped $^{14}CO_2$ was eluted from the central vial with 5 ml of Rotiszint 22 (Roth GmbH & Co. Chemische Fabrik, Karlsruhe, Federal Republic of Germany) and counted for radioactivity.

Radioactive labeling of plasmid-encoded proteins. The following protocol was received from Stan Tabor (Harvard Medical School, Boston, Mass.). Cells of *E. coli* K38 containing both pGP1-2 and the pT7-recombinant plasmid were grown in LB medium under ampicillin and kanamycin selection. At an A_{590} of 0.5, 0.2 ml of cells was centrifuged, washed in 5 ml of M9 medium (25), recentrifuged, and suspended in 1.0 ml of M9 medium supplemented with 20 μg of thiamine per ml and 18 proteinogenic amino acids (each 0.01%, without cysteine and methionine). The cells were shaken at 30°C for 60 min, and then the temperature was increased to 42°C. After 15 min, 10 μl of a rifampin stock solution (20 mg/ml in methanol) was added to a final concentration of 200 μg/ml, and the cells were left at 42°C for 10 min. Thereafter the temperature was decreased to 30°C for 20 min, and samples were then pulsed with 10 μCi of L-[^{35}S]methionine (Amersham; cell labeling grade) for 5 min at room temperature and centrifuged. The cell pellet was suspended in 120 μl of cracking buffer (60 mM Tris hydrochloride, pH 6.8, 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue). The samples were heated to 95°C for 3 min and loaded onto an SDS-polyacrylamide gel as described by Laemmli (23).

RESULTS

Transposon mutants of pJP4. Of 1,000 kanamycin-resistant clones which had Tn*5* stably integrated in their genome, we isolated 8 mutants that were unable to grow on 2,4-D. All of these strains carried the transposon on plasmid pJP4, as determined by conjugative transfer of kanamycin resistance to *E. coli* HB101 and *A. eutrophus* JMP222. Four 2,4-D$^-$ mutants were still able to grow on 3-CB, thus belonging to mutant class A, B, or F, as described by Don et al. (9). To determine the specific enzyme defects, oxygen uptake of these strains was measured during incubation of whole cells with 2,4-D and 2,4-dichlorophenol as substrates. Two mutant strains were deficient in 2,4-D monooxygenase activity, whereas their 2,4-dichlorophenol hydroxylase was still active when 3-CB was used as an inducer. This fact assigns these strains to mutant class A.

Restriction mapping of pJP4 derivatives of *tfdA* mutants showed that in addition to insertion of Tn*5*, large deletions or rearrangements had occurred which involved *Eco*RI fragments C, E, G, H, and I. Because of their complexity they could not be physically characterized further.

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Cloning of *tfdA*. We first cloned the 21-kilobase *Hin*dIII fragment of pJP4 in pVK101. A restriction map of the fragment is shown in Fig. 1. The recombinant plasmid pVJH21 was transferred by direct conjugation on selective medium to *tfdA*-defective transposon mutants, using *E. coli* S17-1 as a mobilizing donor strain. Colonies appeared in mating experiments on 2,4-D medium after 14 days and showed wild-type growth. The system was further used to test subcloned fragments for complementation or marker rescue of Tn*5* mutants. *Sac*I fragments from pVJH21 were shotgun-ligated with the broad-host-range vector plasmid pGSS33, and *E. coli* LE392 was transformed with the DNA. Recombinant plasmids were isolated, identified, brought into strain S17-1 by transformation, and subsequently transferred to the transposon mutants. Plasmids such as pGJS3 containing a 3-kilobase *Sac*I fragment of pJP4/pVJH21 were able to restore 2,4-D metabolism in both mutants.

Expression in *A. eutrophus* JMP222. Strain JMP222 is able to grow on phenol as a carbon source by using chromosomally located genes for a meta-cleavage pathway, but cannot grow on PAA. On the other hand, we know from oxygen uptake assays with induced cultures of strain JMP134 that the *tfdA*-encoded 2,4-D monooxygenase can also convert unsubstituted PAA to phenol. Thus, growth on PAA was chosen as a test for expression of *tfdA* in strain JMP222. The 3-kb *Sac*I fragment was subcloned by using the broad-host-range vector pKT231, and hybrid plasmids pKJS31 and pKJS32 (inserts in opposite orientations) were subsequently mobilized from *E. coli* S17-1 to *A. eutrophus* JMP222. Colonies growing on PAA (Paa$^+$) appeared after 3 to 4 days; no difference was observed in the behavior of clones carrying the different hybrid plasmids. All tested Paa$^+$ colonies were kanamycin resistant, whereas from colonies selected on kanamycin several had lost the ability to grow on PAA.

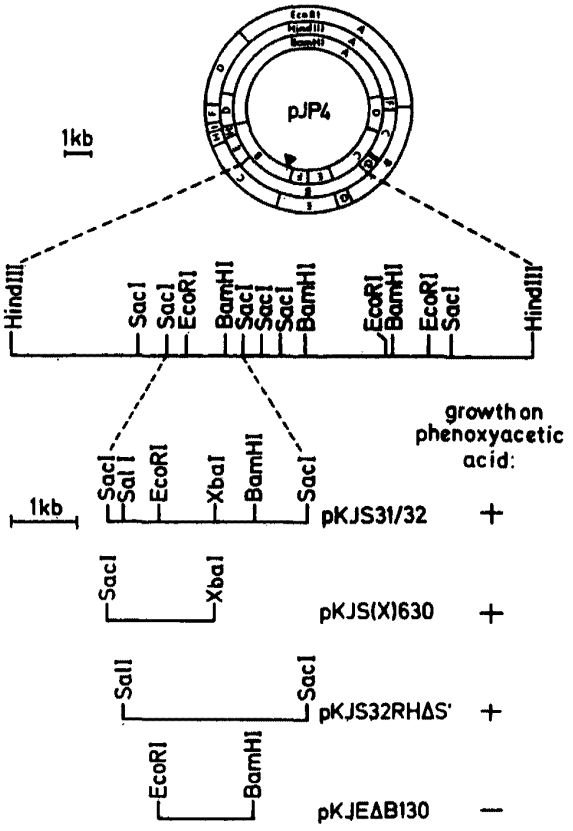


FIG. 1. Cloning and deletion analysis of *tfdA*. Hybrid plasmids containing fragments of pJP4 cloned in broad-host-range vector pKT231 were transferred from *E. coli* S17-1 to *A. eutrophus* JMP222 by conjugation. Transconjugants carrying an intact *tfdA* gene were able to grow on PAA as a carbon source. The localization of *tfdA* on pJP4 is indicated by the solid triangle.

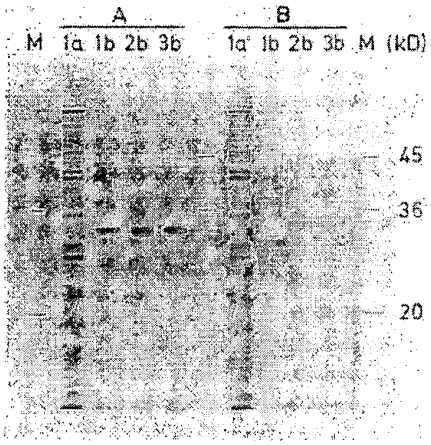


FIG. 2. Expression of gene *tfdA* by T7 RNA polymerase. Three DNA fragments able to confer PAA degradation on *A. eutrophus* JMP222 (1, 2.8-kb *Sac*I-*Sal*I; 2, 2.1-kb *Bam*HI-*Sal*I; 3, 1.4-kb *Xba*I-*Sal*I) were cloned in T7 promoter plasmids pT7-5 (A) and pT7-6 (B), yielding constructions with both orientations of the insert in relation to the T7 promoter. Strains of *E.coli* K38 carrying a heat-inducible T7 RNA polymerase gene on plasmid pGP1-2 and one of the hybrid pT7 plasmids were used for specific labeling of plasmid-encoded proteins: gene expression was induced by heat, the host RNA polymerase was inhibited with rifampin, and cells were pulsed with L-[^{35}S]methionine as described in Materials and Methods (lanes b). For labeling of total cell protein, samples were treated omitting both heat induction and rifampin addition (lanes a). Proteins were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and revealed by autoradiography. Transcription from the *Xba*I to the *Sal*I site resulted in expression of a 32,000-dalton protein (panel A), whereas no protein was expressed from opposite orientations (panel B). M, Molecular weight markers (transferred from Coomassie blue-stained gel).

Expression in *Pseudomonas* sp. strain B13. As the side-chain cleavage enzyme encoded by *tfdA* accepts differently substituted and unsubstituted PAAs as substrates, we supposed that introduction of *tfdA* into *Pseudomonas* sp. strain B13, which is able to degrade 4-chlorophenol (29), could extend the degradative capacity of strain B13 to 4-chlorophenoxyacetic acid. Plasmids pKJS31 and pKJS32 were transferred by conjugation from *E. coli* S17-1 to *Pseudomonas* sp. strain B13. As expected, transconjugants were able to grow on 4-chlorophenoxyacetic acid, and, in addition, they could also use PAA as a growth substrate.

Deletion analysis. We used the growth of strain JMP222 on PAA as an appropriate system to test constructions with several deletions for the expression of *tfdA*. Small DNA fragments were deleted between unique restriction sites on pKT231 and the *Sac*I insert. Up to the *Xba*I site from one

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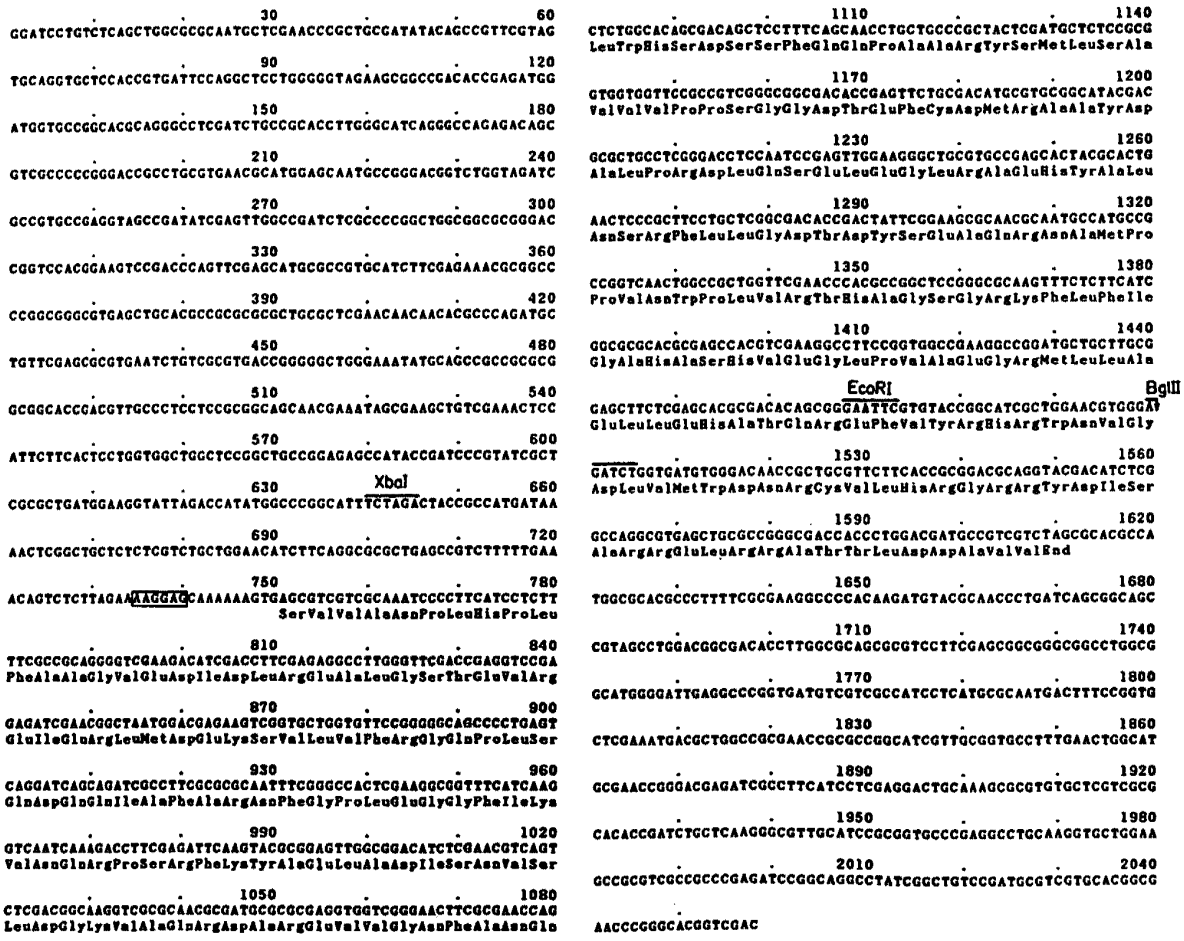


FIG. 3. Nucleotide sequence of the 2.06-kb *Bam*HI-*Sal*I fragment of pJP4 encoding the 2,4-D monooxygenase gene *tfdA*. The coding strand of the DNA is presented in the 5'-to-3' direction along with the deduced amino acid sequence of the only possible open reading frame. The *Bgl*II cleavage site where the omega fragment was inserted is indicated at nucleotide 1500. A putative Shine-Dalgarno sequence in front of the coding region is enclosed in a box.

end and to the *Sal*I site from the other end, deletions could be made without loss of degradative activity (Fig. 1), whereas deletion of the smaller *Eco*RI-*Sac*I fragment resulted in inactivation of *tfdA*.

Specific labeling of *tfdA* gene product. All fragments able to confer PAA degradation on strain JMP222 were subcloned from pKT231 in T7 RNA polymerase-promoter plasmids pT7-5 and pT7-6, yielding constructions with both orientations of the insert in relation to the promoter. Plasmid-encoded proteins were labeled with L-[^{35}S]methionine, separated by SDS-polyacrylamide gel electrophoresis, and revealed by autoradiography. Figure 2 shows the expression of a single protein from all constructions in which the T7 RNA polymerase reads from the *Xba*I to the *Sal*I restriction site. The protein molecular weight was determined to be about 32,000 by comparison with standard protein markers.

Expression of 2,4-D monooxygenase in *E. coli*. pTJS'X535 is a hybrid plasmid of pT7-5 and the *Sal*I-*Xba*I fragment of pJP4 (Fig. 1). *E. coli* K38, containing both pGP1-2 and pTJS'X535, was able to release $^{14}CO_2$ from 2,4-D labeled in position 2 of the acetate side chain. Kinetic assays show that 70% of total trapped radioactivity is released within the first hour of incubation, thus indicating a high level of expression of 2,4-D monooxygenase from the T7 RNA promoter.

Nucleotide sequence of the cloned *tfdA* DNA. The 2.8-kb *Sac*I-*Sal*I fragment shown in Fig. 1 was subcloned in replicative forms of both phages M13tg130 and M13tg131. To create a series of deletions for primed dideoxynucleotide sequencing, unidirectional digestion with exonuclease III was carried out by the method of Henikoff (20). For this purpose, recombinants from M13tg130 were cleaved by *Sal*I and *Pst*I, whereas recombinants from M13tg131 were cleaved by *Sac*I and *Bam*HI. Clones containing plasmids with deletions were analyzed by restriction endonuclease cleavage of replicative forms, and appropriate deletions were chosen for sequencing. The nucleotide sequence was determined by the method of Sanger et al. (28).

Translation of the sequence shown in Fig. 3, from the *Xba*I to the *Sal*I site where *tfdA* gene activity is expressed, reveals one possible open reading frame which corresponds

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in its length to a protein size of 32,000 as determined from electrophoresis of labeled *tfdA* gene product.

Insertion mutagenesis of cloned *tfdA*. As 2,4-D monooxygenase activity was directly correlated with expression of a protein of 32,000 molecular weight and with presence of an intact open reading frame of 861 base pairs, we assumed that insertion of transcriptional and translational stop signals at a *Bgl*II site situated within this reading frame would result in expression of a truncated protein and in inactivation of 2,4-D monooxygenase. We excised the omega fragment from pDOC37 with *Bam*HI and inserted it into a *Bgl*II-cleaved pTJS'X535. Specific labeling by the method of Tabor and Richardson (34) as described above revealed a protein of 29,000 molecular weight (Fig. 4), which matches the predicted size calculated from a shortened open reading frame of 768 base pairs. No enzymatically active 2,4-D monooxygenase was expressed from the omega-mutagenized plasmid, as shown by radioisotopic 2,4-D assay (Table 1).

TABLE 1. Correlation of 2,4-D monooxygenase activity, protein length, protein length, and open reading frames in a *tfdA*-pT7 hybrid plasmid and its insertion derivative

Plasmid	Enzyme activity[a] (kcpm)	Mol mass of protein[b] (daltons)	Open reading frame[c] (base pairs)
pT7-5	0.3		
pTJS'X535	70	32,000	861
pTJS'X535omega	0.4	29.000	768

[a] Measured release of $^{14}CO_2$.
[b] Determined by SDS-polyacrylamide gel electrophoresis.
[c] From GTG (base 748) to first in-frame stop.

DISCUSSION

In this report we describe the cloning and characterization of gene *tfdA* from *A. eutrophus* JMP134, which encodes the first enzymatic step in the degradation of 2,4-D. The identity of the cloned gene was confirmed by the following criteria: (i) complementation of *tfdA*-defective transposon mutants; (ii) expression of PAA degradative capacity in *A. eutrophus* JMP222; and (iii) expression of 2,4-D side chain cleavage in *E. coli*.

We have identified the protein encoded by *tfdA*, and we have determined the nucleotide sequence of the cloned gene. One possible open reading frame was found, which starts at base 748 with a GTG codon and ends at base 1608 in front of a TAG stop codon. The translational start was confirmed by determination of the first 16 N-terminal amino acids of the purified protein (data not shown). Upstream of base 748, a Shine-Dalgarno sequence can be identified (base 735). Estimation of a molecular weight of 31,000 to 33,000 for the *tfdA* gene product by SDS-polyacrylamide gel electrophoresis is in agreement with the molecular weight of 32,171 predicted from the 861-nucleotide open reading frame. Functional correlation of the nucleotide sequence and enzyme activity has been shown by insertion mutagenesis.

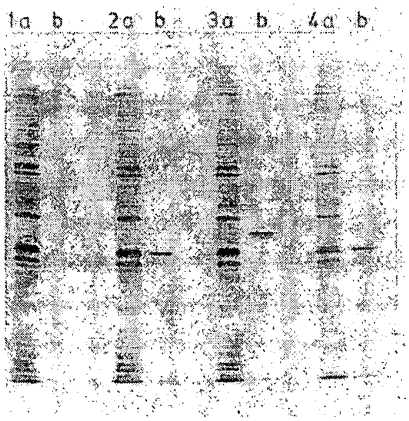


FIG. 4. Omega mutagenesis of gene *tfdA*. The omega fragment, which carries transcriptional and translational stop signals, was inserted at a *Bgl*II site situated within the coding region of *tfdA* on plasmid pTJS'X535. Gene expression was induced using the T7 RNA polymerase-promoter system. Protein was labeled with L-[^{35}S]methionine, loaded onto a 12.5% SDS-polyacrylamide gel, and revealed by autoradiography. No protein is expressed from the vector plasmid pT7-5 (lanes 1). A truncated *tfdA* gene product is expressed from both omega-mutagenized plasmids pTJS'X535omega (lanes 2 and 4), whereas a full-length protein is expressed from pTJS'X535 (lanes 3). Samples were labeled after heat induction and addition of rifampin (lanes b) or omitting induction and rifampin addition (lanes a).

It is now of interest to elucidate the regulation of 2,4-D degradation in strain JMP134. The promoter which is obviously present on the cloned *tfdA* fragment could serve as a tool for the search of a regulatory gene. Sequence analysis shows an AT-rich area upstream of the coding region, and insertion of a DNA fragment into the *Xba*I site results in poor expression of *tfdA* if a foreign promoter is not provided on the insert (data not shown). Promoter structures can therefore be expected around that area.

Interestingly, gene *tfdA* is located at a distance of 13 kb from the gene cluster encoding the hydroxylation and *meta*-cleavage of 2,4-dichlorophenol. A similar separation of catabolic genes into upper and lower pathway gene clusters has also been observed on the NAH (naphthalene degradation) plasmid (39) and the TOL (toluene degradation) plasmid (14). This finding provides additional support for the "module" theory, which postulates an evolution of degradative pathways by sequential assembly of distinct parts of the pathway (14). Together with conjugation, this provides gram-negative bacteria with an enormous flexibility for the evolution of new degradative functions.

Another trait which gene *tfdA* has in common with other catabolic genes is the broad substrate specificity of the encoded enzyme. It is able to use 2,4-D, 2-methyl-4-chlorophenoxyacetic acid, 4-chlorophenoxyacetic acid, and PAA as substrates. A variety of related compounds which have not yet been tested may perhaps be metabolized as well. Similar broad specificities have been demonstrated for xylene oxidase (19, 26). We can expect therefore that the cloned *tfdA* gene will be useful in the construction of novel pathways. Potential uses of gene *tfdA* may be seen not only in the field of bacterial degradative functions, but also in genetic engineering of plants. The metabolism of 2,4-D by some plant species has already been reported (31, 38). We achieved tolerance of a *Nicotiana silvestris* haploid cell suspension culture against 2,4-D by adapting it to higher concentrations of the synthetic growth hormone (40). As gene *tfdA* specifies the side chain cleavage of 2,4-D, the capacity of plant cells to detoxify chlorinated PAA herbicides could be increased by the transformation of cells with *tfdA*, followed by expression of an enzymatically active protein. Thus, another bacterial gene would be available, in

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addition to the prevalently used neomycin phosphotransferase gene, to function as a selectable marker gene for plant genetic experiments, and perhaps transformed cells can be regenerated to whole, herbicide-resistant plants.

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Links

The two enantiospecific dichlorprop/alpha-ketoglutarate-dioxygenases from Delftia acidovorans MC1--protein and sequence data of RdpA and SdpA.

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Two alpha-ketoglutarate-dependent dioxygenases carrying enantiospecific activity for the etherolytic cleavage of racemic phenoxypropionate herbicides [(RS)-2-(2,4-dichlorophenoxy)propionate and (RS)-2-(4-chloro-2-methylphenoxy)propionate] from Delftia acidovorans MC1 were characterized with respect to protein and sequence data. The (S)-phenoxypropionate/alpha-ketoglutarate-dioxygenase (SdpA) appeared as a monomeric enzyme with a molecular weight of 32 kDa in the presence of SDS. N-terminal sequences revealed relationship to alpha-ketoglutarate-dependent taurine dioxygenase (TauD) and to 2,4-dichlorophenoxyacetate/alpha-ketoglutarate-dioxygenase (TfdA). The (R)-phenoxypropionate/alpha-ketoglutarate-dioxygenase (RdpA) referred to 36 kDa in the presence of SDS and to 108 kDa under native conditions. Internal sequences of fragments obtained after digestion made evident relationship to TfdA and TauD. Two-dimensional electrophoretic separation resulted in the resolution of up to 3 individual spots with almost identical molecular weights but different isoelectric points with both RdpA and SdpA. The structural differences of these isoenzyme forms are not yet clear.

PMID: 12501996 [PubMed - indexed for MEDLINE]

Related Links

Localization and characterization of two novel genes encoding stereospecific dioxygenases catalyzing 2(2,4-dichlorophenoxy) propionate cleavage in Delftia acidovorans [Appl Environ Microbiol. 2004]

Purification and characterization of two enantioselective alpha-ketoglutarate-dependent dioxygenases, RdpA and SdpA, from Sphingomonas herbicidovorans MH. [Appl Environ Microbiol. 2006]

Physiological and genetic characteristics of two bacterial strains utilizing phenoxypropionate and phenoxyacetate he[illegible] Res. 2001]

Structural basis for the enantiospecificities of R- and S-specific phenoxypropionate/alpha-ketoglutarate dioxygenases[Protein Sci. 2006]

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Acta Biotechnol. **23** (2003) 1, 3–17

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 μM and 30 μM, 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 μM and 22 μM, 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 μM, 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 Fe^{2+} and ascorbate; other divalent cations showed inhibitory effects to different extents. Activity was completely extinguished within 2 min in the presence of 100 μM 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

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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], *Rhodoferax* 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; $CaCl_2 \times 6\ H_2O$, 11; $MgSO_4 \times 7\ H_2O$, 142; $FeSO_4 \times 7\ H_2O$, 10; $CuSO_4 \times 5\ H_2O$, 1.57; $MnSO_4 \times 4\ H_2O$, 1.23; $ZnSO_4 \times 7\ H_2O$, 0.88; $Na_2MoO_4 \times 2\ H_2O$, 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 $OD_{700} = 2$, 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 × 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.

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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 dioxygenase 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

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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_4; 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 ≤ 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 $OD_{700} = 1$ 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). Densidometric 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

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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

	(*R*)-specific enzyme			(*S*)-specific enzyme		
Purification step	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

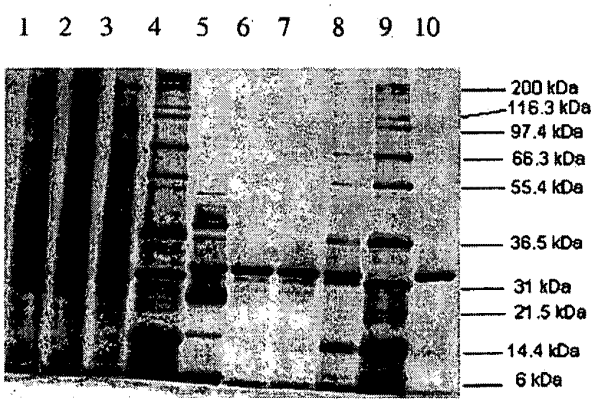


Fig. 1. Purification of (*S*)-2,4-DP/α-KG dioxygenase from *D. acidovorans* MC1
Protein samples were analysed by gel electrophoresis on a 12% polyacrylamide gel and visualised by silver staining.
1: Crude extract, 2: Supernatant after the treatment with 0.15% protamine sulphate, 3: Fraction precipitated 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.

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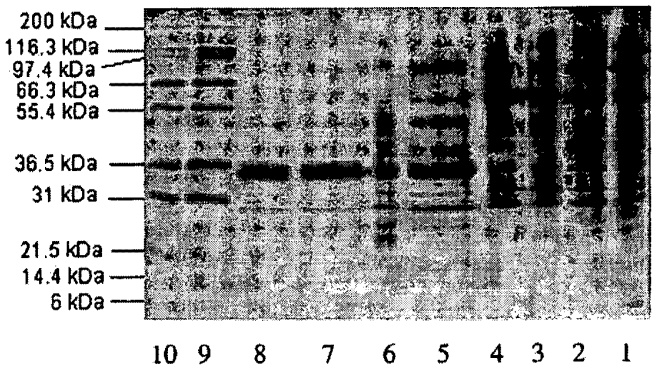


Fig. 2. Purification of (*R*)-2,4-DP/α-KG dioxygenase from *D. acidovorans* MC1
Protein samples were analysed by gel electrophoresis on a 12% polyacrylamide gel and visualised by silver staining.
1: Crude extract, 2: Supernatant after treatment with 0.15% protamine sulphate, 3+4:Pooled fractions with (*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).

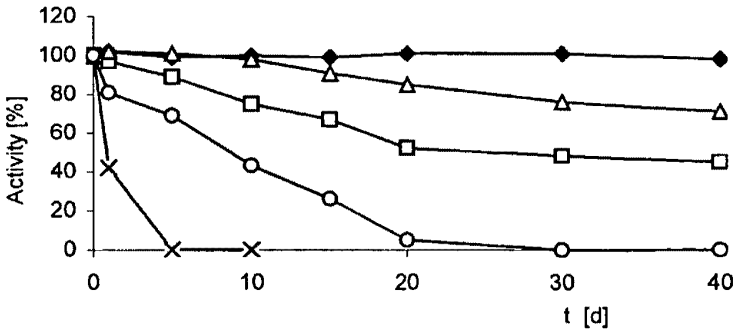


Fig. 3. Stability of the (*R*)-2,4-DP/α-KG dioxygenase (after HIC) at different storage conditions
(○) 4 °C without addition, (×) 4 °C after the addition of 50 μM ascorbic acid, (□) 4 °C after the addition of 1% BSA, (Δ) 4 °C after the addition of 3.4 M ammonium sulphate, (◆) − 20 °C without addition.

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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 μM. With regard to the physical parameters, the temperature optimum was determined as 30 °C with the *R*-specific and 25 °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.

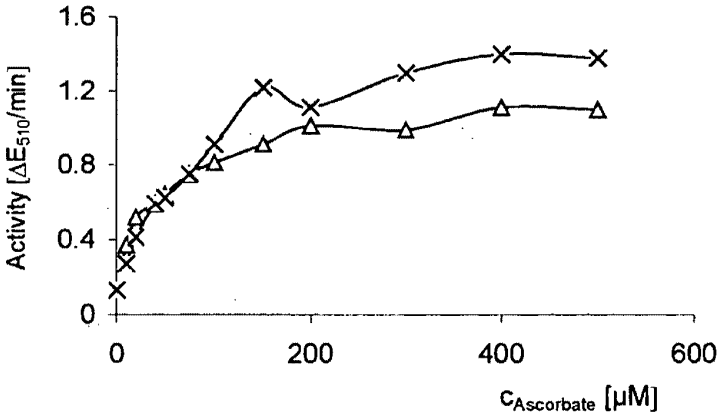


Fig. 4. Effect of ascorbic acid on the activity of 2,4-DP/α-KG dioxygenases (×) *S*-specific enzyme, (Δ) *R*-specific enzyme.

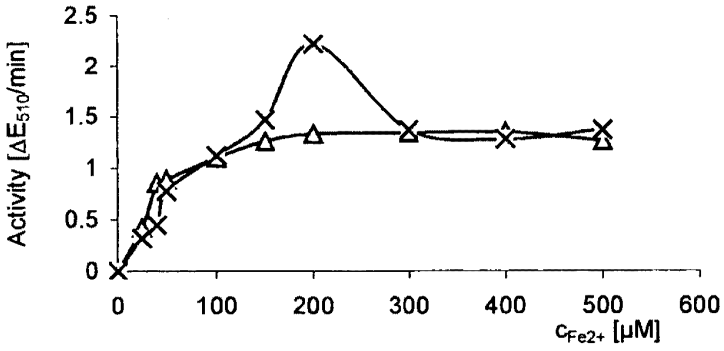


Fig. 5. Activation of 2,4-DP/α-KG dioxygenases by ferrous ions (×) S-specific enzyme, (Δ) *R*-specific enzyme.
The enzyme activity was determined using standard assay conditions.

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Tab. 2. Substrate specificity and kinetic parameters of *(R)*-2,4-DP/α-KG dioxygenase

Substrate (concentration range studied)		K_m [μM]	k_{kat} [min^{-1}]	k_{kat}/K_m	[%]
(R)-2,4-DP	(20–200 μM)	54.9 ± 4.4	55.2	1	/ (100)
(S)-2,4-DP	(100–5000 μM)	–	–	–	
(R)-2,4-MCPP	(10–200 μM)	30 ± 1.2	34.4	1.15	/ (115)
(S)-2,4-MCPP	(20–600 μM)	261 ± 40	3.8	0.02	/ (2)
2,4-D	(20–2000 μM)	1305 ± 120	7.6	0.006	/ (0.6)
2,4-DB	(100–2000 μM)	–	–	–	
3-Phenoxypropionic acid	(100–2000 μM)	–	–	–	
(R,S)-2-(2,4,5-Trichlorophenoxy)propionic acid	(10-200 μM)	130.8 ± 9.3	50	0.382	/ (38)
(R,S)-2-(*m*-Chlorophenoxy)propionic acid	(20-400 μM)	176.2 ± 4.2	34.4	0.195	/ (19)
(R,S)-2-(4-Chlorophenoxy)propionic acid	(10-200 μM)	131 ± 0.4	65.2	0.498	/ (50)
α-Ketoglutarate	(2.5–50 μM)	27.8 ± 3.8	20	0.719	/ (100)
α-Ketobutyrate	(10–5000 μM)	–	–	–	
α-Ketoadipate	(10–5000 μM)	–	–	–	
α-Ketoisovalerate	(10–5000 μM)	–	–	–	
α-Ketovalerate	(10–5000 μM)	–	–	–	
Pyruvate	(10–10000 μM)	–	–	–	

(– = no activity detected)

All experiments were performed at 30 °C in 10 mM imidazol buffer (pH 6) containing 200 μM ascorbate, 200 μM $(NH_4)_3Fe(SO_4)_2$, 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

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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 ≥ 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 [μM]	k_{kat} [min^{-1}]	k_{kat}/K_m	[%]
(R)-2,4-DP (50–500 μM)	–	–	–	
(S)-2,4-DP (20–200 μM)	49 ± 9.1	50	1.02	/ (100)
(R)-2,4-MCPP (20–1000 μM)	–	–	–	
(S)-2,4-MCPP (10–100 μM)	21.8 ± 5	46	2.11	/ (207)
2,4-D (50–200 μM)	122.8 ± 2	36	0.29	/ (29)
2,4-DB (100–2000 μM)	–	–	–	
3-Phenoxypropionic acid (100–2000 μM)	–	–	–	
(R,S)-2-(2,4,5-Trichlorophenoxy)propionic acid (100–2000 μM)	–	–	–	
(R,S)-2-(*m*-Chlorophenoxy)propionic acid (20–200 μM)	11.5 ± 6.4	15	0.13	/ (13)
(R,S)-2-(4-Chlorophenoxy)propionic acid (10–100 μM)	68.3 ± 9.8	17	0.25	/ (25)
α-Ketoglutarate (2.5–50 μM)	24.1 ± 7.6	1.7	34	/ (100)
α-Ketobutyrate (10–5000 μM)	–	–	–	
α-Ketoadipate (10–5000 μM)	–	–	–	
α-Ketoisovalerate (10–5000 μM)	–	–	–	
α-Ketovalerate (10–5000 μM)	–	–	–	
Pyruvate (10–10000 μM)	–	–	–	

(– = no activity detected)

All experiments were performed at 25 °C in 10 mM imidazol buffer (pH 6) containing 200 μM ascorbate, 200 μM $(NH_4)_3Fe(SO_4)_2$, 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.

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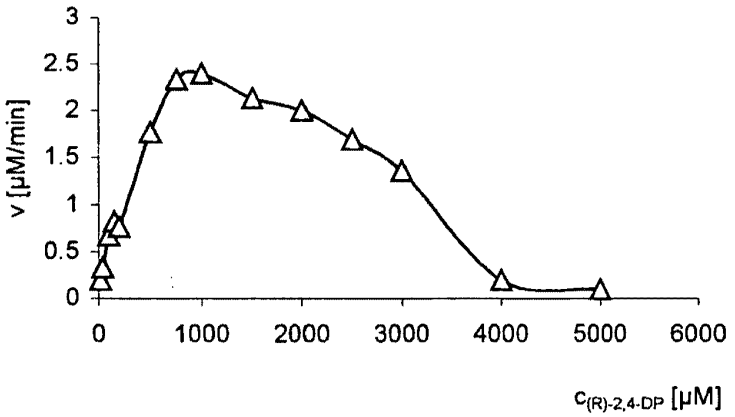


Fig. 6 a. Effect of (*R*)-2,4-DP on the activity of (*R*)-2,4-DP/α-KG dioxygenase
The assay contained 0.055 µM enzyme.

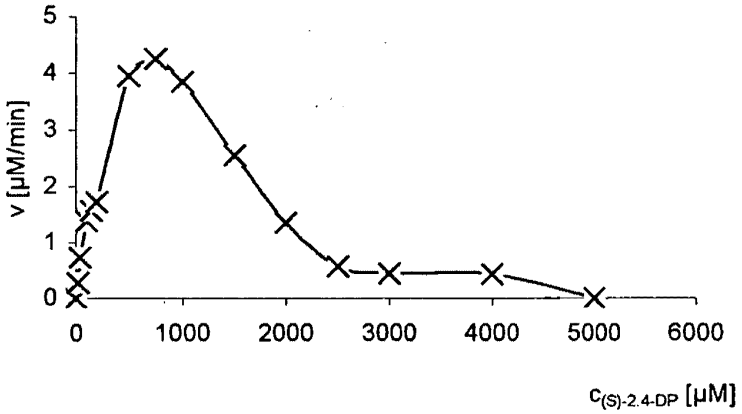


Fig. 6 b. Effect of (*S*)-2,4-DP on the activity of (*S*)-2,4-DP/α-KG dioxygenase
The assay contained 0.055 µM enzyme.

Both enzymes exhibited excess substrate inhibition. According to the data in Figs. 6 a 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.

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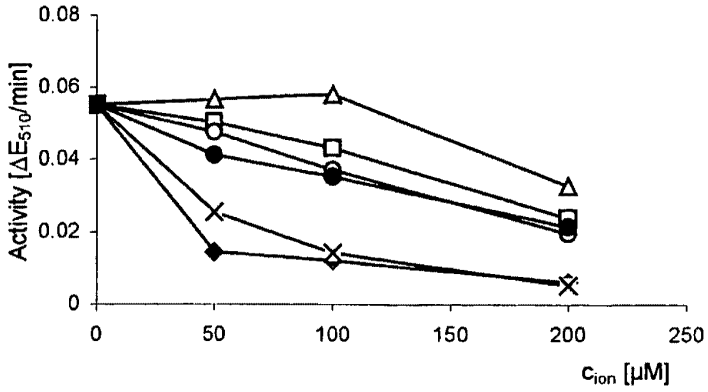


Fig. 7 a. Metal ion-dependent inactivation of (*R*)-2,4-DP/α-KG dioxygenase
The enzyme activity was determined by using standard assay condition in the presence of various metal ions.
(Δ) Mg^{2+}, (□) Ni^{2+}, (○) Mn^{2+}, (●) Co^{2+}, (×) Zn^{2+}, (◆) Cu^{2+}.

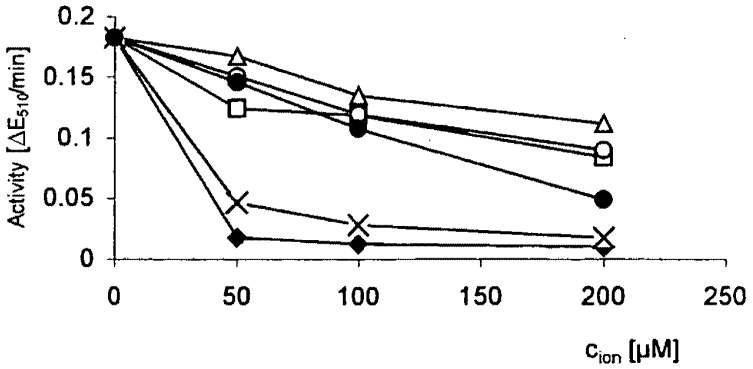


Fig. 7 b. Metal ion-dependent inactivation of (*S*)-2,4-DP/α-KG dioxygenase
The enzyme activity was determined by using standard assay condition in the presence of various metal ions.
(Δ) Mg^{2+}, (□) Ni^{2+}, (○) Mn^{2+}, (●) Co^{2+}, (×) Zn^{2+}, (◆) Cu^{2+}.

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 μM with the *R*-specific but far less than 50 μM with the *S*-specific enzyme. The other ions tested exerted weaker effects. In addition, the enzyme was inhibited by DEPC. The application of 100 μM led to an almost complete loss of activity within 2 min with both enzymes; lower concentrations behaved more gradually (Figs. 8 a,b).

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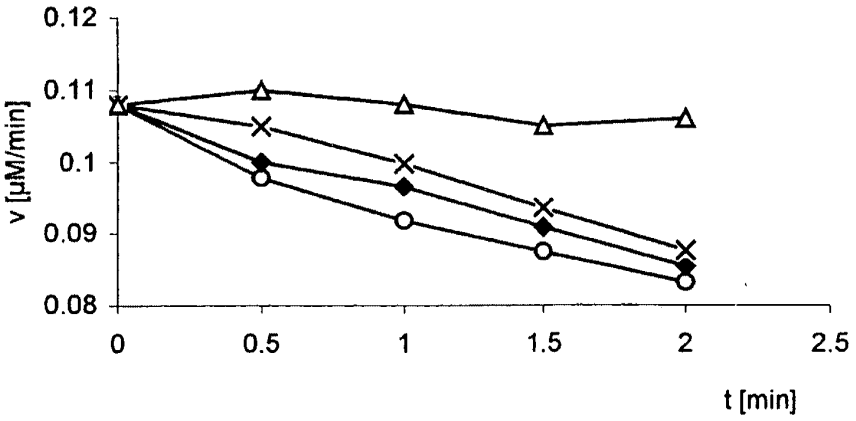


Fig. 8 a. Inactivation of (*R*)-2,4-DP/α-KG dioxygenase with DEPC
(○) 100 µM DEPC, (◆) 50 µM DEPC, (×) 20 µM DEPC, (Δ) 0 µM DEPC.

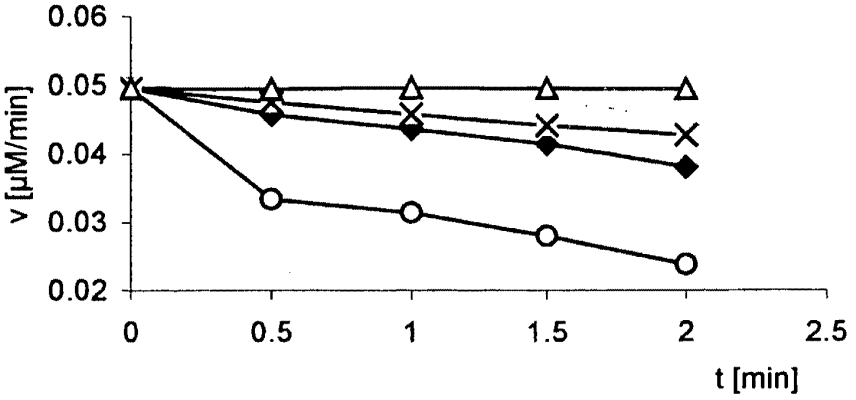


Fig. 8 b. Inactivation of (*S*)-2,4-DP/α-KG dioxygenase with DEPC
(○) 100 µM DEPC, (◆) 50 µM DEPC, (×) 20 µM DEPC, (Δ) 0 µM DEPC.

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*-enantio-

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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 Fe^{2+} 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*)-MCPP or (*R*)-MCPP, 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, *Rhodoferax* 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.

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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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Bayer EX1005-0460 (1 of 2)

IAP07Rec'd PCT 24 MAR 2008

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on **March 21, 2008**.

Jay M. Sanders, Patent Attorney

INFORMATION DISCLOSURE STATEMENT
Patent Application
Docket No. DAS-115XC1
Serial No. 11/587,893

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry Wright, Justin Lira, Don Merlo, Nicole Hopkins
Serial No.	:	11/587,893
Filed	:	October 27, 2006
For	:	Novel Herbicide Resistance Genes

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

INFORMATION DISCLOSURE STATEMENT

Sir:

In accordance with 37 CFR §1.97 and §1.98, the references listed on the attached Form PTO/SB/08 are hereby submitted for consideration by the Patent Office. Copies of the listed U.S. Patents are not provided pursuant to 37 CFR 1.98(a)(2)(ii).

It is respectfully requested that the listed references be made of record.

Applicants respectfully assert that the substantive provisions of 37 CFR §§1.97 and 1.98 are met by the foregoing statements.

Though no fees are believed to be required for this paper, the Patent Office is hereby authorized to charge any fees required by this paper to Deposit Account No. 19-0065.

Respectfully submitted,

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

JMS/mrc
Attachments: Form PTO/SB/08

J:\DAS\DAS-115XC1\PTO\IDS\IDS.doc/DNB/mrc

IAP07Rec'd PCT 22 MAY 2008

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to the Mail Stop - PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on **May 20, 2008**.

Jay M. Sanders, Patent Attorney

Patent Application
Docket No. DAS-115XC1
Serial No. 11/587,893

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry Wright, Justin Lira, Donald Merlo, Nicole Arnold
Serial No.	:	11/587,893
Filed	:	October 27, 2006
For	:	Novel Herbicide Resistance Genes

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

TRANSMITTAL LETTER

Sir:

The above-identified patent application was filed with an unsigned Declaration (37 CFR 1.63) and Power of Attorney form. Transmitted herewith is a fully executed Declaration (37 CFR 1.63) and Power of Attorney form for the subject application.

Attached hereto please find a Supplemental Application Data Sheet which amends the Applicant Information in the above-referenced patent application. Entry of the Supplemental Application Data Sheet into the record is respectfully requested. **Applicants respectfully request a Corrected Filing Receipt** be issued showing the updated name (due to marriage) of the Inventor.

J:\DAS\DAS-115XC1\PTO\Trsn-Ltr-Dec-POA.doc/DNB/mrc

Please charge the surcharge of $130.00 to Deposit Account No. 19-0065. The Commissioner is hereby authorized to charge any additional fees which may be required to Deposit Account No. 19-0065. Two copies of this letter are enclosed.

Respectfully submitted,

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

JMS/mrc
Attachments: executed Declaration and Power of Attorney
Supplemental Application Data Sheet

Bayer EX1005-0463 (1 of 2)

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to the Mail Stop - PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on **May 20, 2008**.

Jay M. Sanders, Patent Attorney

Patent Application
Docket No. DAS-115XC1
Serial No. 11/587,893

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry Wright, Justin Lira, Donald Merlo, Nicole Arnold
Serial No.	:	11/587,893
Filed	:	October 27, 2006
For	:	Novel Herbicide Resistance Genes

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

TRANSMITTAL LETTER

Sir:

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J:\DAS\DAS-115XC1\PTO\Trsn-Ltr-Dec-POA.doc/DNB/mrc

Please charge the surcharge of $130.00 to Deposit Account No. 19-0065. The Commissioner is hereby authorized to charge any additional fees which may be required to Deposit Account No. 19-0065. Two copies of this letter are enclosed.

Respectfully submitted,

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

JMS/mrc
Attachments: executed Declaration and Power of Attorney
Supplemental Application Data Sheet

Bayer EX1005-0465 (1 of 2)

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to the Mail Stop - PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on **May 20, 2008**.

Jay M. Sanders, Patent Attorney

Patent Application
Docket No. DAS-115XC1
Serial No. 11/587,893

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Terry Wright, Justin Lira, Donald Merlo, Nicole Arnold
Serial No.	:	11/587,893
Filed	:	October 27, 2006
For	:	Novel Herbicide Resistance Genes

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

TRANSMITTAL LETTER

Sir:

The above-identified patent application was filed with an unsigned Declaration (37 CFR 1.63) and Power of Attorney form. Transmitted herewith is a fully executed Declaration (37 CFR 1.63) and Power of Attorney form for the subject application.

Attached hereto please find a Supplemental Application Data Sheet which amends the Applicant Information in the above-referenced patent application. Entry of the Supplemental Application Data Sheet into the record is respectfully requested. **Applicants respectfully request a Corrected Filing Receipt** be issued showing the updated name (due to marriage) of the Inventor.

Bayer EX1005-0466 (1 of 2)

Please charge the surcharge of $130.00 to Deposit Account No. 19-0065. The Commissioner is hereby authorized to charge any additional fees which may be required to Deposit Account No. 19-0065. Two copies of this letter are enclosed.

Respectfully submitted,

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

JMS/mrc
Attachments: executed Declaration and Power of Attorney
Supplemental Application Data Sheet

Bayer EX1005-0467 (1 of 2)

Docket Ref. **63,364A**

DECLARATION AND POWER OF ATTORNEY USA/PCT

As a below named inventor, I hereby declare that:

(a) My residence and Citizenship are as stated below my name. My P.O. (mailing) address is the same as my residence unless otherwise stated.

(b) I verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter that is embraced by and for which a patent is sought on the invention entitled: **NOVEL HERBICIDE RESISTANCE GENES**
and the specification of which: ☐ is attached hereto (_____).
(check one) ☐ was filed on _____ as (_____).
Application No. _____
and was amended on _____

(c) I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

(d) I acknowledge my duty under 37 CFR 1.56 to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in 37 CFR 1.56(b). If this application is a continuation-in-part application, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 CFR 1.56(b) that became available between the filing date of the prior application from which priority is claimed in part (f) below, and the national or PCT international filing date of this application.

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

PRIOR FOREIGN APPLICATION(S)			PRIORITY CLAIMED	CERTIFIED COPIES INCL.
Number	**Country or PCT**	**Day/Month/Year Filed**		
			☐	☐

☐ Additional claims for benefit are attached.

(f) I hereby Claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below, or under 35 U.S.C. §120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States of America listed below:

US or PCT Appln. Serial No.	Filing Date	Status at Application Filing Date
US 60/567,052	April 30, 2004	
PCT/US2005/014737	May 2, 2005	

☐ Additional claims for benefit are attached.

I hereby appoint the attorney(s) and/or agent(s) at the following Customer No. to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Address all correspondence to appointed counsel at:

23557

This appointment, including the right to delegate this appointment, shall also apply to the same extent it is applicable under the laws of the United States of America to any proceedings established by the Patent Cooperation Treaty.

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 are 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.

Inventor(s):

At: Indianapolis, Indiana 46268 United States of America
this 15 day of November, 2006
Effective as of May 2, 2005
Signature: _____
Full Name: **Terry R. Wright**
Residence: **14162 Charity Chase Circle**
Westfield, Indiana 46074
Country: **United States of America**
Citizenship: **United States of America**
P. O. Address: **Same As Residence**

At: Indianapolis, Indiana 46268 United States of America
this 15 day of November, 2006
Effective as of May 2, 2005
Signature: _____
Full Name: **Justin M. Lira**
Residence: **12373 Ensley Drive**
Fishers, Indiana 46038
Country: **United States of America**
Citizenship: **United States of America**
P. O. Address: **Same As Residence**

At: Indianapolis, Indiana 46268 United States of America
this 17 day of November, 2006
Effective as of May 2, 2005
Signature: Donald J Merlo
Full Name: **Donald J. Merlo**
Residence: **11845 Durbin Drive**
Carmel, Indiana 46032
Country: **United States of America**
Citizenship: **United States of America**
P. O. Address: **Same As Residence**

At: Indianapolis, Indiana 46268 United States of America
this 15 day of November, 2006
Effective as of May 2, 2005
Signature: Nicole L Arnold
Full Name: **Nicole L. Arnold**
Residence: **9743 Mill Creek Place**
Carmel, Indiana 46032
Country: **United States of America**
Citizenship: **United States of America**
P. O. Address: **Same As Residence**

☐ Additional names and signatures are attached.

SUPPLEMENTAL APPLICATION DATA SHEET

Docket No. DAS-115XC1

Application Information

Application Number::	11/587,893
Filing Date::	October 27, 2006
Application Type::	Regular (National Stage)
Subject Matter::	Utility
Suggested Classification::	None
Suggested Group Art Unit::	None
CD-ROM or CD-R?::	None
Number of CD disks::	None
Number of copies of CDs::	None
Sequence submission?::	Paper
Computer Readable Form?::	Yes
Number of Copies of CRF::	One
Title::	NOVEL HERBICIDE RESISTANCE GENES
Attorney Docket Number::	DAS-115XC1
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	None
Total Drawing Sheets::	24
Small Entity?::	No
Petition included?::	No
Petition Type::	N/A
Secrecy Order in Parent Appl.?::	No

Bayer EX1005-0469 (1 of 2)

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Unknown
Inventor One Given Name::	Terry
Family Name::	Wright
City of Residence::	Westfield
State or Province of Residence::	IN
Country of Residence::	US
Street of Mailing Address::	14162 Charity Chase Circle
City of Mailing Address::	Westfield
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46074

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Unknown
Inventor Two Given Name::	Justin
Family Name::	Lira
City of Residence::	Fishers
State or Province of Residence::	IN
Country of Residence::	US
Street of Mailing Address::	12373 Ensley Drive
City of Mailing Address::	Fishers
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46038

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Unknown
Inventor Three Given Name::	Donald
Family Name::	Merlo
City of Residence::	Carmel
State or Province of Residence::	IN
Country of Residence::	US
Street of Mailing Address::	11845 Durbin Drive
City of Mailing Address::	Carmel
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46032

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Unknown
Inventor Four Given Name::	Nicole
Family Name::	~~Hopkins~~Arnold
City of Residence::	Indianapolis
State or Province of Residence::	IN
Country of Residence::	US
Street of Mailing Address::	2518 Chaseway Ct.
City of Mailing Address::	Indianapolis
State or Province of mailing address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46268

Representative Information

Representative Customer Number::	000023557

Correspondence Information

Correspondence Customer Number::	000023557
Telephone Number One::	(352) 375-8100
Telephone Number Two::	
Fax Number::	(352) 372-5800
Electronic Mail Address::	jms@slspatents.com

Bayer EX1005-0472 (1 of 2)

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This application is a	National Stage of	PCT/US2005/014737	May 2, 2005
PCT/US2005/014737	An application claiming the benefit under 35 USC 119(e) of	60/567,052	April 30, 2004

Assignee Information

Assignee Name::	Dow AgroSciences LLC
Street of Mailing Address::	9330 Zionsville Road
City of Mailing Address::	Indianapolis
State or Province of Mailing Address::	IN
Country of Mailing Address::	US
Postal or Zip Code of Mailing Address::	46268-1054

Bayer EX1005-0473 (1 of 2)

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 06/27/2008

BCAMPBEL ADJ #00000003 Mailroom Dt: 10/27/2006
Seq No: 99 Sales Acctg Dt: 11/02/2006 190065 11587893
03 FC : 1615 2350.00 CR
04 FC : 1614 400.00 CR

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 06/27/2008

BCAMPBEL	SALE	#00000002	Mailroom Dt:	10/27/2006	190065	11587893	
		01	FC : 1615	2,350.00	DA		
		02	FC : 1614	200.00	DA		

UNITED STATES PATENT AND TRADEMARK OFFICE

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

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
11/587,893	Terry Wright	DAS-115XC1

23557
SALIWANCHIK LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
PO BOX 142950
GAINESVILLE, FL 32614-2950

INTERNATIONAL APPLICATION NO.	
PCT/US2005/014737	
I.A. FILING DATE	PRIORITY DATE
05/02/2005	04/30/2004

CONFIRMATION NO. 7868
371 FORMALITIES LETTER



Date Mailed: 06/30/2008

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as an Elected Office (37 CFR 1.495):

- Priority Document
- Copy of the International Application filed on 10/27/2006
- Copy of the International Search Report filed on 10/27/2006
- Copy of IPE Report filed on 10/27/2006
- Preliminary Amendments filed on 10/27/2006
- Information Disclosure Statements filed on 03/24/2008
- Biochemical Sequence Diskette filed on 10/27/2006
- Oath or Declaration filed on 05/22/2008
- Biochemical Sequence Listing filed on 10/27/2006
- Request for Immediate Examination filed on 10/27/2006
- U.S. Basic National Fees filed on 10/27/2006
- Priority Documents filed on 10/27/2006
- Power of Attorney filed on 05/22/2008
- Specification filed on 10/27/2006
- Claims filed on 10/27/2006
- Abstracts filed on 10/27/2006
- Drawings filed on 10/27/2006
- Paper nucleotide sequence listings filed on 10/27/2006
- Paper Computer Listings filed on 11/08/2006

The applicant needs to satisfy supplemental fees problems indicated below.

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date. The current oath or declaration does not comply with 37 CFR 1.497(a) and (b) in that it:
  - Inventor name change without supporting documentation
- To avoid abandonment, a surcharge (for late submission of filing fee, search fee, examination fee or oath or declaration) as set forth in 37 CFR 1.492(h) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

FORM PCT/DO/EO/905 (371 Formalities Notice)

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**130** for a Large Entity:

- $**130** Surcharge.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 32 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

BARBARA A CAMPBELL

Telephone: (703) 308-9140 EXT 217

FORM PCT/DO/EO/905 (371 Formalities Notice)

Bayer EX1005-0477 (1 of 2)

PATENT APPLICATION FEE DETERMINATION RECORD
Effective December 8, 2004

Application or Docket Number: 11/587,893

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)	SMALL ENTITY TYPE ☐ RATE	FEE	OR	OTHER THAN SMALL ENTITY RATE	FEE
U.S. NATIONAL STAGE FEES							
BASIC FEE	SMALL ENT. = $ 150	LARGE ENT. = $ 300	BASIC FEE		OR	BASIC FEE	300
EXAMINATION FEE	Satisfies PCT Article 33(1)-(4) = $ 50 / $ 100	All other situations = $ 100 / $ 200	EXAM. FEE			EXAM. FEE	—
SEARCH FEE	U.S. is ISA = $ 50 / $ 100 ALL other countries = $ 200 / $ 400	ALL other situations = $ 250 / $ 500	SEARCH FEE			SEARCH FEE	—
FEE FOR EXTRA SPEC. PGS.	minus 100 =	/ 50 =	X $ 125 =			X $ 250 =	
TOTAL CHARGEABLE CLAIMS (68)	72 minus 20 =	*	X $ 25 =		OR	X $ 50 =	
INDEPENDENT CLAIMS	4 minus 3 =	*	X $ 100 =		OR	X $ 200 =	
MULTIPLE DEPENDENT CLAIM PRESENT		☐	+ $ 180 =		OR	+ $ 360 =	
* If the difference in column 1 is less than zero, enter "0" in column 2			TOTAL		OR	TOTAL	

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE	ADDI-TIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	ADDI-TIONAL FEE
67									
Total	* 67	Minus	** 20	= 47	X $ 25 =		OR	X $ 50 =	2350
Independent	* 4	Minus	*** 3	= 1	X $ 100 =		OR	X $ 200 =	200
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐					+ $ 180 =		OR	+ $ 360 =	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	2550 (RT)

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE	ADDI-TIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	ADDI-TIONAL FEE
Total	*	Minus	**	=	X $ 25 =		OR	X $ 50 =	
Independent	*	Minus	***	=	X $ 100 =		OR	X $ 200 =	
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM ☐					+ $ 180 =		OR	+ $ 360 =	
					TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '20', enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than '3', enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Barbara Campbell, PCT National Stage Division

Bayer EX1005-0478 (1 of 2)

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET
(FOR USE WITH FORM PTO-875)

SERIAL NO. 11/587,893 | FILING DATE 10-27-06
APPLICANT(S)

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
1	1		1			
2		1		1		
3		1		1		
4		1		1		
5		1		1		
6		1		1		
7		1		1		
8		1		1		
9		1		1		
10		1		1		
11		1		1		
12		1		1		
13		1		1		
14		1		1		
15		1		1		
16		1		1		
17		1		1		
18		1		1		
19		1		1		
20		1		1		
21		1		1		
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50		2		1		
TOTAL IND.		↓		↓		↓
TOTAL DEP.		←		←		←
TOTAL CLAIMS						

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
51		2		1		
52		0	e			
53		0		1		
54		1		1		
55		1		1		
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TOTAL IND.	4	↓	4	↓		↓
TOTAL DEP.	68	←	63	←		←
TOTAL CLAIMS	72		67			

PTO - 1360 (REV. 11/04) Barbara Campbell, PCT National Stage U.S. DEPARTMENT of COMMERCE Patent and Trademark Office

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference DAS-115XC1	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2005/014737	International filing date *(day/month/year)* 02 May 2005 (02.05.2005)	Priority date *(day/month/year)* 30 April 2004 (30.04.2004)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant DOW AGROSCIENCES LLC		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☒	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 01 November 2006 (01.11.2006)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Authorized officer Nora Lindner e-mail: pt02@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:
JAY M. SANDERS
SALIWANCHIK, LLOYD & SALIWANCHIK
A PROFESSIONAL ASSOCIATION
P.O. BOX .142950
GAINESVILLE, FL 32614-2950

PCT

REC'D 24 APR 2006
WIPO PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Applicant's or agent's file reference DAS-115XC1	Date of mailing (*day/month/year*) 20 APR 2006 **FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date (*day/month/year*)	Priority date (*day/month/year*)
PCT/US05/14737	02 May 2005 (02.05.2005)	30 April 2004 (30.04.2004)

International Patent Classification (IPC) or both national classification and IPC

IPC: Please See Continuation Sheet
USPC: 536/23.2,23.7;435/419,320.1,468;800/298,300

Applicant

DOW AGROSCIENCES LLC

1. This opinion contains indications relating to the following items:

- ☒ Box No. I — Basis of the opinion
- ☐ Box No. II — Priority
- ☐ Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV — Lack of unity of invention
- ☒ Box No. V — Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI — Certain documents cited
- ☐ Box No. VII — Certain defects in the international application
- ☒ Box No. VIII — Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(*b*) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	28 February 2006 (28.02.2006)	David H. Kruse Telephone No. (571) 272-0547

Form PCT/ISA/237 (cover sheet) (April 2005)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/14737

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☒ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☒ on paper

☒ in electronic form

c. time of filing/furnishing

☒ contained in the international application as filed.

☒ filed together with the international application in electronic form.

☐ furnished subsequently to this Authority for the purposes of search.

3. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Bayer EX1005-0482 (1 of 2)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/14737

Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims 1-68	YES
	Claims NONE	NO
Inventive step (IS)	Claims 1-68	YES
	Claims NONE	NO
Industrial applicability (IA)	Claims 1-68	YES
	Claims NONE	NO

2. Citations and explanations:

Claims 1-68 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest an isolated polynucleotide that encodes an AAD-1 protein having aryloxyalkanoate dioxygenase activity.

Claims 1-68 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

Bayer EX1005-0483 (1 of 2)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/14737

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 47-56, 58 and 63-68 objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims are not fully supported by the description. The application, as originally filed, did not describe: The invention of claim 47 is directed to an isolated polynucleotide that encodes a protein only described by function, especially in view of the limitation "under stringent conditions". Claim 63 is directed to a method of using a crop plant transformed with a nucleic acid encoding an enzyme only described by function.

Bayer EX1005-0484 (1 of 2)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US05/14737

Supplemental Box
In case the space in any of the preceding boxes is not sufficient.

Continuation of IPC:
A01H 1/00(2006.01),5/00(2006.01),5/10(2006.01);C12N 15/52(2006.01),15/31(2006.01),15/82(2006.01)
G01N 33/53(2006.01);C12N 9/02(2006.01)

Bayer EX1005-0485 (1 of 2)

I hereby certify that this correspondence is being electronically filed in the United States Patent and Trademark Office on July 11, 2008.

Jay M. Sanders, Patent Attorney

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT
Patent Application
Docket No. DAS-115XC1
Serial No. 11/587,893

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit	:	1661
Applicants	:	Terry Wright, Justin Lira, Donald Merlo, Nicole Arnold
Serial No.	:	11/587,893
Filed	:	October 27, 2006
Conf. No.	:	7868
For	:	Novel Herbicide Resistance Genes

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

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT
UNDER 37 CFR §§1.97 AND 1.98

Sir:

In accordance with 37 CFR §1.56, the references listed on the attached form PTO/SB/08 are being brought to the attention of the examiner for consideration in connection with the examination of the above-identified patent application. Copies of the cited references are enclosed. However, Applicants have not submitted a copy of the U.S. patent cited on attached Form PTO/SB/08 pursuant to 37 CFR 1.98(a)(2)(ii).

The undersigned hereby certifies that each item of information contained in this Supplemental Information Disclosure Statement was cited in a communication from a foreign patent office in a counterpart foreign application.

It is respectfully requested that the references cited on the attached form PTO/SB/08 be considered in the examination of the subject application and that their consideration be made of record.

Bayer EX1005-0486 (1 of 2)

International Application No. WO 98/02562, cited as F2 on the attached form PTO/SB/08, is written in a foreign language. Applicants are submitting herewith a copy of the European Search Report from corresponding Application No. EP 05771746 which was filed on March 28, 2008. Applicants have also included U.S. Patent No. 7,250,561, cited as U1 on attached form PTO/SB/08. The U.S. patent is cited on the European Search Report as a patent family member of WO 98/02562. Applicants note that although the specification of the WO 98/02562 application is written in a foreign language, an English language Abstract is provided therewith. Applicants respectfully request that the reference be made of record and considered in the examination of the subject application.

Applicants respectfully assert that the substantive provisions of 37 CFR §§1.97 and 1.98 are met by the foregoing statement.

Respectfully submitted,

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

JMS/trt/sl
Attachments: Form PTO/SB/08; copies of references cited therein; copy of European Search Report

Bayer EX1005-0487 (1 of 2)

PTO/SB/08A (08-03)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet	1	of	2

Complete if Known	
Application Number	11/587,893
Filing Date	October 27, 2006
First Named Inventor	Terry Wright
Art Unit	1661
Examiner Name	
Attorney Docket Number	DAS-115XC1

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number - Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	U1	US-7,250,561	07-31-2007	Pallett *et al.*	ALL
	U2	US-			
	U3	US-			
	U4	US-			
	U5	US-			
	U6	US-			
	U7	US-			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] – Number[4] – Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
	F1	WO 98/02562	01-22-1998	Rhone-Poulenc Agrochimie	ALL	
	F2	WO 03/034813	05-01-2003	The United States of America, as Represented by the Secretary of Agriculture	ALL	
	F3					
	F4					
	F5					
	F6					
	F7					

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kind Codes of USPTO Patent Documents at www.uspto.gov or MPEP901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard T.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

This collection of information is required by 37 CFR 1.97 and 1.98. 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 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, 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 (1-800-786-9199) and select option 2.

Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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Substitute for form 1449A/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(use as many sheets as necessary)

Sheet	2	of	2

Complete if Known	
Application Number	11/587,893
Filing Date	October 27, 2006
First Named Inventor	Terry Wright
Art Unit	1661
Examiner Name	
Attorney Docket Number	DAS-115XC1

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No. [1]	Include name of the author (in CAPITAL LETTERS), title of the article, (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	R1	Database EMBL, Accession No. AJ628859, KOHLER, H.P.E. "Sphingomonas herbicidovorans tnpA gene for transposase and rdpA gene for (R)-2-(2,4-dichlorophenoxy)propionate,2-oxoglutarate dioxygenase", March 3, 2004, XP-002474325, pp. 1-2.	
	R2	KOHLER, H.P.E. "Sphingomonas herbicidovorans MH: A versatile phenoxyalkanoic acid herbicide degrader" *Journal of Industrial Microbiology & Biotechnology*, October 1999, pp. 336-340, Vol. 23, No. 4-5	
	R3	Database EMBL, Accession No. AF516751, SCHLEINITZ, K.M. *et al.*, "Rhodoferax sp. P230 R-2,4-dichlorophenoxypropionate dioxygenase (rdpA) gene, complete cds.", August 6, 2002, XP-002474326, pp. 1-2.	
	R4	Database EMBL, Accession No. AF516752, SCHLEINITZ, K.M. *et al.*, "Sphingobium herbicidovorans R-2,4-dichlorophenoxypropionate dioxygenase (rdpA) gene, complete cds.", August 6, 2002, XP-002474327, pp. 1-2.	
	R5	SCHLEINITZ, K.M. "Localization and Characterization of Two Novel Genes Encoding Stereospecific Dioxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate cleavage in *Delftia acidovorans* MC1" *Applied and Environmental Microbiology*, September 2004, pp. 5357-5365, Vol. 70, No. 9.	
	R6	Supplemental European Search Report dated March 28, 2008	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kind Codes of USPTO Patent Documents at www.uspto.gov or MPEP901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard T.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST. 16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

This collection of information is required by 37 CFR 1.97 and 1.98. 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 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, 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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DEMANDE INTERNATIONALE PUBLIEE EN VERTU DU TRAITE DE COOPERATION EN MATIERE DE BREVETS (PCT)

(51) Classification internationale des brevets 6 :
C12N 15/82, 15/53, 15/54, 15/55, 5/10, A01H 5/00

A2

(11) Numéro de publication internationale: **WO 98/02562**

(43) Date de publication internationale: 22 janvier 1998 (22.01.98)

(21) Numéro de la demande internationale: PCT/FR97/01256

(22) Date de dépôt international: 10 juillet 1997 (10.07.97)

(30) Données relatives à la priorité:
96/09137 16 juillet 1996 (16.07.96) FR

(71) Déposant *(pour tous les Etats désignés sauf US)*: RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre Baizet, F-69009 Lyon (FR).

(72) Inventeurs; et
(75) Inventeurs/Déposants *(US seulement)*: PALLETT, Ken [GB/GB]; Rhône-Poulenc Agriculture Ltd., Fyfield Road, Ongar Essex CM5 0HW (GB). DEROSE, Richard [US/FR]; 216, rue de Saint Cyr, F-69009 Lyon (FR). PELISSIER, Bernard [FR/FR]; 49, chemin de Crécy, F-69370 Saint Didier au Mont d'Or (FR). SAILLAND, Alain [FR/FR]; 38, rue Albert Chalinel, F-69009 Lyon (FR).

(74) Mandataire: CHRETIEN, François; Rhône-Poulenc Agrochimie, 14/20, rue Pierre Baizet, F-69009 Lyon (FR).

(81) Etats désignés: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, brevet ARIPO (GH, KE, LS, MW, SD, SZ, UG, ZW), brevet eurasien (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), brevet européen (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), brevet OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Publiée
Sans rapport de recherche internationale, sera republiée dès réception de ce rapport.

(54) Title: CHIMERA GENE WITH SEVERAL HERBICIDE RESISTANT GENES, PLANT CELL AND PLANT RESISTANT TO SEVERAL HERBICIDES

(54) Titre: GENE CHIMERE A PLUSIEURS GENES DE TOLERANCE HERBICIDE, CELLULE VEGETALE ET PLANTE TOLERANTES A PLUSIEURS HERBICIDES

(57) Abstract

The invention concerns: 1.Chimera gene with several herbicide resistant genes, plant cell and plant resistant to several herbicides. 2.The plant is resistant both to several herbicides, particularly to HPPD inhibitors and EPSPS inhibitors and/or to dihalogenohydroxybenzonitriles. 3. Its use for weeding plants with several herbicides.

(57) Abrégé

1. Gène chimère à plusieurs gènes de tolérance herbicide, cellule végétale et plante tolérantes à plusieurs herbicides. 2. La plante est tolérante à la fois à plusieurs herbicides, notamment aux inhibiteurs de l'HPPD et à ceux de l'EPSPS et/ou aux dihalogénohydroxybenzonitriles. 3. Utilisation pour le désherbage de plantes avec plusieurs herbicides.

UNIQUEMENT A TITRE D'INFORMATION

Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

AL	Albanie	**ES**	Espagne	**LS**	Lesotho	**SI**	Slovénie
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EE	Estonie	**LR**	Libéria	**SG**	Singapour		

GENE CHIMERE A PLUSIEURS GENES DE TOLERANCE HERBICIDE, CELLULE VEGETALE ET PLANTE TOLERANTES A PLUSIEURS HERBICIDES

La présente invention a pour objet un gène chimère à plusieurs gènes de tolérance herbicide, une cellule végétale et une plante tolérantes à plusieurs herbicides.

Dans la suite de la description, les herbicides seront désignés selon le nom commun en particulier référencé dans "The Pesticide Manual" 10 th edition par British Crop Protection Council.

On connaît des plantes qui ont été transformées pour être tolérantes à certains herbicides comme notamment les dihalogénohydroxybenzonitriles, en particulier le bromoxynil et l'ioxynil, grâce au gène codant pour la nitrilase dégradant ces herbicides ou encore celles tolérantes aux herbicides inhibiteurs de l'EPSPS notamment le glyphosate, le sulfosate ou la fosametine ou encore aux inhibiteurs de l'acétolactatesynthase (ALS) du type des sulfonylurées ou encore aux inhibiteurs de la dihydro-pteroate synthase tel que l'asulam ou encore aux inhibiteurs de la glutamine synthase tels que le glufosinate.

On connaît certains herbicides tels que les isoxazoles décrites notamment dans les demandes de brevets français 95 06800 and 95 13570 et notamment l'isoxaflutole, herbicide sélectif du maïs, les dicétonitriles tels que ceux décrits dans les demandes européennes 0 496 630, 0 496 631, en particulier la 2-cyano-3-cyclopropyl-1-(2-SO_2 CH_3-4-CF_3 phényl) propane-1,3-dione et la 2-cyano-3-cyclopropyl-1-(2-SO_2 CH_3-4-2,3 Cl_2 phényl) propane-1, 3-dione, les tricétones décrites dans les demandes européennes 0 625 505 et 0 625 508, en particulier la sulcotrione ou encore celles décrites dans l'USP 5 506 195, ou encore les pyrazolinates. De plus le gène codant pour l'HPPD conférant une tolérance à ces derniers herbicides a été isolé et des plantes transgéniques le contenant obtenues montrant une tolérance significative et font l'objet des demandes françaises non publiées N°95/06800, 95/13570 et 96/05944.

Cependant la pratique agricole montre que les agriculteurs aiment disposer pour le traitement des plantes, et notamment des cultures, d'associations d'herbicides en particulier pour répondre à différents problèmes de désherbage dûs aux limites du spectre des herbicides pris isolément. Il peut être en outre intéressant de disposer d'un gène de marquage de sélection associé à un gène de tolérance herbicide. Il y a donc un besoin de plantes et notamment de cultures présentant une tolérance à plusieurs herbicides, de préférence au moins deux ou trois .

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Il a maintenant été découvert qu'on pouvait conférer à une cellule végétale et à une plante un tolérance herbicide multiple.

La présente invention a d'abord pour objet un gène chimère comprenant au moins deux gènes chimères élémentaires contenant chacun, dans le sens de la transcription, des éléments de régulation nécessaires à sa transcription dans les plantes c'est à dire au moins une séquence de régulation promotrice, au moins une partie codante hétérologue comprenant une séquence codante codant pour une enzyme conférant aux plantes la tolérance à un herbicide et au moins une séquence de régulation terminatrice ou de polyadénylation.

Comme séquence codante, on peut notamment utiliser toutes celles connues pour conférer la tolérance de plantes à certains inhibiteurs telles que:

- celle de l'EPSPS pour la tolérance au glyphosate, au sulfosate ou à la fosamétine, notamment celles de la protéine mutée ou non, on peut citer notamment les brevets;

- USP 4 535 060, EP 115 673, USP 4 769 061, USP 5 094 945; USP 4 971 908, USP 5 145 783, EP 293 358; EP 378 985 , WO 91/04323; WO 92 044 449; WO 92 06201. Dans la suite ce type de gène sera désigné par séquence ou gène "EPSPS".

On peut également citer la glyphosate oxydoréductase (cf WO 92/ 000 377) enzyme de détoxification du glyphosate.

- celle du gène de la nitrilase de *Klebsiella sp.* pour la tolérance aux dihalogénobenzonitriles décrite dans l'USP 4 810 648 et en particulier celui issu de *Klebsiella ozaenae*, qui sera désigné dans la suite par gène ou séquence "OXY".

- celle de l'HPPD telle décrite dans les demandes françaises non publiées N°95/06800, 95/13570 et 96/05944 citées ci-dessus. Cette HPPD peut être de toute nature.

Plus particulièrement cette séquence peut être d'origine bactérienne, telle que notamment le genre *Pseudomonas* ou encore d'origine végétale, telle que notamment de plante monocotylédone ou dicotylédone, notamment d'*Arabidopsis* ou d'ombellifères comme par exemple la carotte (*Daucus carota*). Elle peut être native ou sauvage ou éventuellement mutée tout en gardant fondamentalement une propriété de tolérance herbicide contre les inhibiteurs de l'HPPD, tels que les herbicides de la famille des isoxazoles ou de celle des tricétones ou des pyrazolinates.

D'autres séquences peuvent être utilisées:

- celle de la phosphinotrycine acétyl transférase ou celle de la glutamine synthase pour la tolérance au glufosinate (cf EP 0 242 236)

- celle de la dihydroptéroate synthase pour la tolérance à l'asulam (cf EP 0 369 367)

- celle de l'ALS pour la tolérance aux sulfonylurées

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- celle de la Protoporphyrogen oxydase ("protox") pour la tolérance aux herbicides de la famille des diphényléthers tels que l'acifluorfen ou l'oxyfluorfen ou celle des oxadiazoles tels que l'oxadiazon ou l'oxadiargyl ou celle des imides cycliques tels que le chlorophthalim ou celle des phénylpyrrazoles tel que le TNP ou celles des pyridines et les phénopylates et analogues carbamates (cf WO 95/34659).

De préférence l'un des gènes chimères contient une séquence codante de l'HPPD. Dans ce cas l'autre ou les autres séquences peuvent être quelconques et notamment choisies dans le groupe mentionné ci-dessus. De préférence les autres séquences sont choisies dans le groupe comprenant le gène de la nitrilase de tolérance aux dihalogénohydroxybenzonitriles et un gène EPSPS.

Les gènes chimères selon l'invention peuvent en outre contenir des gènes codant pour des propriétés autres que de tolérance herbicides tels que par exemple des gènes de résistance aux insectes, tels que ceux de type *Bacillus thurigensis* conférant une résistance à divers représentants de la famille des coléoptères, des lépidoptères, ou encore des gènes de résistance aux nématodes, des gènes de résistance aux maladies fongiques ou microbiennes, ou encore des gènes conférant des propriétés agronomiques telles que les gènes des diverses desaturases intervenant dans la production des acides gras. On peut citer en particulier celui de la delta -6 desaturase décrit dans la demande internationale WO 93/06712.

Comme séquence de régulation promotrice on peut utiliser toute séquence promotrice d'un gène s'exprimant naturellement dans les plantes en particulier un promoteur d'origine bactérienne, virale ou végétale tel que, par exemple, celui d'un gène de la petite sous-unité de ribulose-biscarboxylase (RuBisCO) ou de celui d'un gène de l'α tubuline (Demande européennne EP n° 0 652 286), ou encore d'un gène de virus de plante tel que, par exemple, celui de la mosaïque du choux fleur (CaMV 19S ou 35S), mais tout promoteur convenable connu peut être utilisé. De préférence on a recours à une séquence de régulation promotrice qui favorise la surexpression de la séquence codante, tel que par exemple, celle comprenant au moins un promoteur d'histone tel que décrit dans la demande européenne EP 0507698.

Selon l'invention, on peut également utiliser, en association avec la séquence de régulation promotrice, d'autres séquences de régulation, qui sont situées entre le promoteur et la séquence codante, telles que des activateurs de trancription "enhancer", comme par exemple l'activateur de translation du virus etch du tabac (TEV) décrit dans la demande WO87/07644, ou des peptides de transit, soit simples, soit doubles, et dans ce cas éventuellement séparés par une séquence intermédiaire, c'est à dire comprenant, dans le sens de la transcription, une séquence codant pour un peptide de transit d'un gène végétal codant pour une enzyme à localisation plastidiale, une partie de séquence de la partie mature N terminale d'un gène végétal codant pour une enzyme à localisation plastidiale, puis une séquence codant pour un second peptide de transit d'un gène végétal codant pour une enzyme à localisation plastidiale, constituée d'une partie de séquence de la partie mature N

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terminale d'un gène végétal codant pour une enzyme à localisation plastidiale, tels que décrit dans la demande européenne n° 0 508 909.

Comme séquence de régulation terminatrice ou de polyadénylation, on peut utiliser toute séquence correspondante d'origine bactérienne, comme par exemple le terminateur nos d'*Agrobacterium tumefaciens*, ou encore d'origine végétale, comme par exemple un terminateur d'histone tel que décrit dans la demande européenne EP n° 0 633 317.

L'invention a encore pour objet une cellule végétale, de plantes monocotylédones ou dicotylédones, notamment des cultures, tolérante à aux moins deux herbicides dont au moins un est un inhibiteur de l'HPPD. Cette cellule peut contenir au moins deux gènes chimères comprenant chacun une séquence codant pour la tolérance à un herbicide et dont l'un comprend une séquence codant pour l'HPPD. Les deux gènes chimères peuvent être soit portés par un même vecteur, soit chacun sur un vecteur différent, soit encore apportés tels quels par introduction dans la cellule par des moyens physiques ou physico-chimiques, par exemple par microinjection, électroporation ou bombardement, selon des méthodes en soi connues.

L'invention a encore pour objet une plante transformée tolérante à au moins deux herbicides dont un est inhibiteur de l'HPPD. Cette plante peut être obtenue soit par croisement d'au moins deux plantes contenant chacune un gène codant pour la tolérance à un herbicide, soit par régénération d'une cellule selon l'invention, telle que décrite ci-dessus. Les plantes peuvent être des monocotylédones ou dicotylédones, notamment des cultures, grandes cultures telles que par exemple mais de manière non limitative pour les dicotyledones le tabac, le coton, le colza, le soja, la betterave, et pour les monocotyledones le maïs et les céréales à paille, ou en core des cultures maraichères ou florales.

L'invention a encore pour objet un procédé d'obtention de plantes à tolérance herbicide multiple par trangénèse végtale, caractérisé en ce que:

- dans une première étape, on insère dans plusieurs cellules respectivement un des gènes élémentaires contenant chacun des éléments de régulation nécessaires à sa transcription dans les plantes et une séquence codante codant pour une enzyme conférant aux plantes la tolérance à un herbicide, et que
- ensuite les plantes sont croisées pour obtenir des plantes à tolérance multiple.

L'invention a encore pour objet un autre procédé d'obtention de plantes à tolérance herbicide multiple par trangénèse végétale, une première étape comportant l' intégration dans des cellules végétales d'au moins deux gènes de tolérances à un herbicide dont au moins un est un inhibiteur de l'HPPD, la seconde étape compremant la régénération de la plante à partir des cellules transformées selon l'invention.

La transformation peut être obtenue par tout moyen connu approprié, amplement décrit dans la littérature spécialisée et notamment les demandes et brevets cités dans la présente demande.

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Une série de méthodes consiste à bombarder des cellules ou des protoplastes avec des particules auxquelles sont accrochées les séquences d'ADN. Selon l'invention ces ADN peuvent être portés par les mêmes particules ou par des bombardements différents. Une autre série de méthodes consiste à utiliser comme moyen de transfert dans la plante un gène chimère inséré dans un plasmide Ti d'*Agrobacterium tumefaciens* ou Ri *d'Agrobacterium rhizogenes*.

D'autres méthodes peuvent être utilisées telles que la micro-injection ou l'électroporation.

L'homme de métier fera le choix de la méthode appropriée en fonction de la nature de la plante, notamment de son caractère monocotylédone ou dicotylédone.

On a observé que des plantes transformées selon l'invention présentent une tolérance significative aux inhibiteurs de l'hydroxy phényl pyruvate dioxygénase tels que certains herbicides récents tels que les isoxazoles décrites notamment dans les demandes de brevets français 9506800 and 95 13570 et notamment du 4-[4-CF3-2-(méthylsulfonyl) benzoyl]-5-cyclopropyl isoxazole,ou "isoxaflutole", herbicide sélectif du maïs, les dicétonitriles tels que ceux décrits dans les demandes européennes 0 496 630, 0 496 631, en particulier la 2-cyano-3-cyclopropyl-1-(2-SO_2 CH_3-4-CF_3 phényl) propane-1,3-dione et la 2-cyano-3-cyclopropyl-1-(2-SO_2 CH_3-4-2,3 Cl_2 phényl) propane-1, 3-dione, les tricétones décrites dans les demandes européennes 0 625 505 et 0 625 508, en particulier la sulcotrione et les pyrazinolates. Ces mêmes plantes selon l'invention présentent une tolérance significative à d'autres herbicides tels que par exemple les dihalogéno benzonitriles, notamment le bromoxynil et l'ioxynil, le glyphosate et ses analogues, le glufosinate

La présente invention a encore pour objet les plantes régénérées à partir des cellules transformées. La régénération est obtenue par tout procédé approprié qui dépende de la nature de l'espèce, comme par exemple décrit dans les demandes ci-dessus. Les plantes selon l'invention peuvent encore être obtenues par croisement de parents, chacun d'eux portant l'un des gènes de tolérance herbicide décrites.

L'invention a enfin pour objet un procédé de désherbage de plantes, notamment de cultures, à l'aide d'un herbicide de ce type, caractérisé en ce qu'on applique cet herbicide sur des plantes transformées selon l'invention, tant en présemis, en prélevée qu'en postlevée de la culture. Par herbicide au sens de la présente invention on entend une matière active herbicide seule ou associée à un additif qui modifie son efficacité comme par exemple un agent augmentant l'activité (synergiste) ou limitant l'activité (en anglais safener).

Bien entendu, pour leur application pratique, les herbicides ci-dessus sont associée de manière en soi connue aux adjuvants de formulations utilisés habituellement en agrochimie

Selon l'invention l'un des gènes de tolérance herbicides présents dans les plantes peut être utilisé comme marqueur de sélection, soit in vitro, soit in vivo .

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Les différents aspects de l'invention seront mieux compris à l'aide des exemples expérimentaux ci-dessous.

Exemple 1: Isolement du gène de l'HPPD de *P. fluorescens* A32 .

A partir de la séquence en acides aminés de l'HPPD de *Pseudomonas sp.* P.J. 874 (publié par Rüetschi U. et al. 1992. Eur. J. Biochem. 205: 459-466), on déduit la séquence de différents oligonucléotides pour amplifier par PCR une partie de la séquence codante de l'HPPD de *P. fluorescens* A32 (isolée par McKellar, R.C. 1982. J. Appl Bacteriol. 53:305-316). Un fragment d'amplification du gène de cette HPPD a été utilisé pour cribler une banque génomique partielle de *P. fluorescens* A32 et ainsi isoler le gène codant pour cette enzyme.

A) Préparation de l'ADN génomique de *P. fluorescens* A32.

La bactérie a été cultivée dans 40 ml de milieu minimum M63 (KH_2PO_4 13,6g/l, $(NH_4)_2SO_4$ 2g/l, $MgSO_4$ 0,2g/l, $FeSO_4$ 0,005 g/l pH7 plus L-tyrosine 10mM comme seule source de carbone) à 28°C pendant 48 heures.

Après lavage, les cellules sont reprises dans 1 ml de tampon de lyse (tris HCl 100 mM pH 8,3, NaCl 1,4 M et EDTA 10 mM) et incubées 10 minutes à 65°C. Après un traitement au phénol/chloroforme (24/1) et un traitement au chloroforme, les acides nucléiques sont précipités par addition d'un volume d'isopropanol puis repris dans 300 µl d'eau stérile et traités à la RNAse 10 µg/ml final. L'ADN est de nouveau traité au phénol/chloroforme, chloroforme et reprécipité par addition de 1/10 de volume d'acétate de sodium 3M pH5 et 2 volumes d'éthanol. L'ADN est ensuite repris dans de l'eau stérile et dosé.

B) Choix des oligonucléotides et synthèses.

A partir de la séquence en acides aminés de l'HPPD de *Pseudomonas sp.* P.J. 874 on choisit cinq oligonucléotides, deux dirigés dans le sens NH_2 terminal de la protéine vers le COOH terminal de la protéine et trois dirigés dans le sens inverse (voir figure 1). Le choix a été dicté par les deux règles suivantes:

-une extrémité 3' de l'oligonucléotide stable, c'est à dire au moins deux bases sans ambiguité.

-une dégénérescence la plus faible possible.

Les oligonucléotides choisis ont les séquences suivantes:

P1: 5'TA(C/T)GA(G/A)AA(C/T)CCIATGGG3'

P2: 5'GA(G/A)ACIGGICCIATGGA3'

P3: 5'AA(C/T)TGCATIA(G/A)(G/A)AA(C/T)TC(C/T)TC3'

P4: 5'AAIGCIAC(G/A)TG(C/T)TG(T/G/A)ATICC3'

P5: 5'GC(C/T)TT(A/G)AA(A/G)TTICC(C/T)TCICC3'

Ils ont été synthétisés sur le synthétiseur "Cyclone plus DNA Synthesizer" de marque MILLPORE.

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Avec ces cinq oligonucléotides par PCR les fragments d'amplification que l'on doit obtenir théoriquement d'après la séquence SEQ ID N°1 ont les tailles suivantes:

avec les amorces P1 et P3 --------> environ 690 bp
avec les amorces P1 et P4 --------> environ 720 bp
avec les amorces P1 et P5 --------> environ 1000 bp
avec les amorces P2 et P3 --------> environ 390 bp
avec les amorces P2 et P4 --------> environ 420 bp
avec les amorces P2 et P5 --------> environ 700 bp

C) Amplification d'une partie codante de l'HPPD de *P. fluorescens* A32.

Les amplifications ont été faites sur un appareil PCR PERKIN ELMER 9600 et avec la Taq polymérase PERKIN ELMER avec son tampon dans les conditions standards, c'est à dire pour 50µl de réaction il y a les dNTP à 200µM, les primers à 20µM, la Taq polymérase 2,5 unités et l' ADN de *P. fluorescens* A32 2,5 µg.

Le programme d'amplification utilisé est, 5 min à 95°C puis 35 cycles <45 sec 95°C, 45 sec 49°C, 1 min 72°C> suivis de 5 min à 72°C.

Dans ces conditions, tous les fragments d'amplification obtenus ont une taille compatible avec les tailles théoriques données au-dessus, ce qui est une bonne indication de la spécificité des amplifications.

Les fragments d'amplifications obtenus avec les jeux d'amorces P1/P4, P1/P5 et P2/P4 sont ligués dans pBSII SK(-) après digestion de ce plasmide par Eco RV et traitement à la terminal transférase en présence de ddTTP comme décrit dans HOLTON T.A. and GRAHAM M.W. 1991. N.A.R. vol 19, n°5 p1156.

Un clone de chacun des trois types est séquencé partiellement; ceci permet de confirmer qu'on a bien amplifié dans les trois cas une partie de la région codante de l'HPPD de *P. fluorescens* A32. Le fragment P1/P4 est retenu comme sonde pour cribler une banque génomique partielle de *P. fluorescens* A32 et isoler le gène complet de l'HPPD.

D) Isolement du gène.

Par Southern on montre qu'un fragment de 7 Kbp après digestion de l'ADN de *P. fluorescens* A32 par l'enzyme de restriction BamHI s'hybride avec la sonde HPPD P1/P4. On a donc fait digérer 400µg d'ADN de *P. fluorescens* A32 par l'enzyme de restriction BamHI et purifier sur gel d'agarose les fragments d'ADN faisant environ 7Kbp.

Ces fragments sont ligués dans pBSII SK(-), lui-même digéré par Bam HI et déphosphorylé par traitement à la phosphatase alcaline. Après transformation dans *E. coli* DH10b, la banque génomique partielle est criblée avec la sonde HPPD P1/P4.

Un clone positif a été isolé et appelé pRP A. Sa carte simplifiée est donnée figure 2. Sur cette carte est indiqué la position de la partie codante du gène HPPD. Elle est composée de 1077 nucléotides qui codent pour 358 acides aminés (voir SEQ ID N° 1). L'HPPD de *P.*

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fluorescens A32 présente une bonne homologie en acides aminés avec celle de *Pseudomonas* sp. strain P.J. 874, il y a en effet 92% d'identité entre ces deux protéines (voir figure 3).

Exemple 2 : Construction de deux gènes chimères avec une séquence HPPD.

Pour conférer la tolérance de plantes aux herbicides inhibant l'HPPD, on construit deux gènes chimères:

Le premier consiste à mettre la partie codante du gène de l'HPPD de *P. fluorescens* A32 sous le contrôle du promoteur double histone (Demande de Brevet européen N° 0 507 698) suivi du Tobacco etch virus translational enhancer (TEV) (pRTL-GUS (Carrington and Freed, 1990; J. Virol. 64: 1590-1597)) avec le terminateur du gène de la nopaline synthase. L'HPPD sera alors localisée dans le cytoplasme.

Le deuxième sera identique au premier, à ceci près qu'entre l'activateur de translation TEV et la partie codante de l'HPPD, on intercale le peptide de transit optimisé (OTP) (Demande européenne EP n° 0 508 909). L'HPPD sera alors localisée dans le chloroplaste.

A) Construction du vecteur pRPA-RD-153:

- pRPA-RD-11 Un dérivé de pBS-II SK(-) (Stratagene catalog #212206) contenant le site de polyadenylation de la nopaline synthase (NOS polyA) (Demande européennne n° 0 652 286) est cloné entre les sites *KpnI* et *SalI*. Le site *KpnI* est transformé en un site NotI par traitement avec la T4 ADN polymerase I en présence de 150 µM de deoxynucleotide triphoshates puis ligation avec un linker NotI (Stratagene catalog #1029). Ainsi on obtient une cassette de clonage NOS polyA .

- pRPA-RD-127: Un dérivé de pRPA-BL-466 (Demande européenne EP n° 0 337 899) cloné dans pRPA-RD-11 créant une cassette d'expression du gène *oxy* et contenant le promoteur de la petite sous unité de la ribulose-biscarboxylase:

" promoter (SSU) - *oxy* gene - NOS polyA"

Pour créer ce plasmide, pRPA-BL-488 a été digéré avec XbaI et HindIII pour isoler un fragment de 1.9 kpb contenant le promoteur SSU et le gène *oxy*, qui a été ligué dans le plasmide pRPA-RD-11 digéré avec des enzymes compatibles.

- pRPA-RD-132: C'est un dérivé de pRPA-BL-488 (Demande européenne EP n° 0 507 698) cloné dans pRPA-RD-127 avec création d'une cassette d'expression du gène *oxy* avec le promoteur double histone:

" promoteur double histone - *oxy* gène - NOS polyA "

Pour fabriquer ce plasmide, pRPA-BL-466 est digéré par HindIII, traité par la Klenow puis redigéré avec NcoI. Le fragment de 1.35 kbp purifié contenant le promoteur double histone H3A748 est ligué avec le plasmide pRPA-RD-127 qui avait été digéré par XbaI, traité Klenow et redigéré par NcoI.

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- pRPA-RD-153: C'est un derivé de pRPA-RD-132 contenant l'activateur de translation du virus etch du tabac (TEV). pRTL-GUS (Carrington and Freed, 1990; J. Virol. 64: 1590-1597) est digéré avec *NcoI* et *EcoRI* et le fragment de 150 bp est ligué dans pRPA-RD-132 digéré avec les mêmes enzymes. Donc on a créé une cassette d'expression contenant le promoteur:

"double histone promoteur - TEV -*oxy* u - NOS polyA"

B) Construction du vecteur pRPA-RD-185:

pUC19/GECA: Un dérivé de pUC-19 (Gibco catalog #15364-011) contenant de nombreux sites de clonage. pUC-19 est digéré avec *EcoRI* et ligué avec l'oligonucleotide linker 1:

Linker 1: AATTGGGCCA GTCAGGCCGT TTAAACCCTA GGGGGCCCG
CCCGGT CAGTCCGGCA AATTTGGGAT CCCCCGGGC TTAA

Le clone sélectionné contient un site *EcoRI* suivi du polylinker qui contient les sites suivants: *EcoRI, ApaI, AvrII, PmeI, SfiI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI* et *HindIII*.

pRPA-RD-185: c'est un dérivé de pUC19/GECA contenant un polylinker modifié. pUC19/GECA est digéré par HindIII et ligué avec l'oligonucleotide linker 2:

Linker 2: AGCTTTTAAT TAAGGCGCGC CCTCGAGCCT GGTTCAGGG
AAATTA ATTCCGCGCG GGAGCTCGGA CCAAGTCCC TCGA

Le clone sélectionné contient un site *HindIII* site au milieu du polylinker qui contient maintenant les sites suivants: *EcoRI, ApaI, AvrII, PmeI, SfiI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI, HindIII, PacI, AscI XhoI* et *EcoNI*.

C) Construction du vecteur pRP T:

- pRP O: un dérivé de pRPA-RD-153 contenant une cassette d'expression de l'HPPD, promoteur double histone - TEV - gène HPPD - terminateur Nos. Pour fabriquer pRP O, pRPA-RD153 est digéré par Hind III, traité par la Klenow puis redigéré par NcoI pour enlever le gène *oxy* et le remplacer par le gène HPPD sorti du plasmide pRP A par digestion BstEII, traitement par la Klenow et redigestion par NcoI.

- pRP R: pour l'obtenir le plasmide pRP O a été digéré par PvuII et SacI, le gène chimère a été purifié puis ligué dans pRPA-RD-185 lui-même digéré par PvuII et SacI.

- pRP T: il a été obtenu par ligation du gène chimère sorti de pRP R après digestion par SacI et HindIII dans le plasmide pRPA-BL 150 alpha2 digéré par les mêmes enzymes (Demande européenne EP n° 0 508 909).

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Le gène chimère du vecteur pRP T a donc la structure suivante:

Promoteur double histone	TEV	Région codante de l'HPPD	Terminateur nos

D) Construction du vecteur pRP V

- pRP P: c'est un dérivé de pRPA-RD-7 (Demande européennne EP n° 0 652 286) contenant le peptide de transit optimisé suivi du gène de l'HPPD. Il a été obtenu par ligation de la partie codante de l'HPPD sorti de pRP A par digestion BstEII et NcoI, traitement à la Klenow et du plasmide pRPA-RD-7 lui-même digéré SphI et AccI et traité à la DNAse polymérase T4.

- pRP Q: un dérivé de pRPA-RD-153 contenant une cassette d'expression de l'HPPD, promoteur double histone - TEV - OTP - gène HPPD - terminateur Nos. Pour le construire le plasmide pRPA-RD-153 est digéré par Sal I, traité par la Klenow puis redigéré par NcoI pour enlever le gène *oxy* et le remplacer par le gène HPPD sorti du plasmide pRP P par digestion BstEII, traitement par la Klenow et redigestion par NcoI.

- pRP S: pour l'obtenir, le plasmide pRP Q a été digéré par PvuII et SacI pour sortir le gène chimère qui a été ligué dans pRPA-RD-185 lui-même digéré par PvuII et SacI.

- pRP V: il a été obtenu par ligation du gène chimère sorti de pRP S après digestion par SacI et HindIII dans le plasmide pRPA-BL 150 alpha2 (Demande européennne EP n° 0 508 909).

Le gène chimère du vecteur pRP Q a donc la structure suivante:

Promoteur double histone	TEV	OTP	Région codante de l'HPPD	Terminateur nos

Exemple 3 : Transformation du tabac industriel PBD6.

Afin de déterminer l'efficacité de ces deux gènes chimériques, ceux-ci ont été transférés dans du tabac industriel PBD6 selon les procédures de transformation et de régénération déjà décrites dans la demande européenne EP n° 0 508 909.

1) Transformation:

Le vecteur est introduit dans la souche non oncogène d'*Agrobacterium* EHA 101 (Hood et al,1987) porteuse du cosmide pTVK 291(Komari et al,1986). La technique de transformation est basée sur la procédure de Horsh R. et al. (1985) Science, 227, 1229-1231.

2) Régénération:

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La régénération du tabac PBD6 (provenance SEITA France) à partir d'explants foliaires est réalisée sur un milieu de base Murashige et Skoog (MS) comprenant 30g/l de saccharose ainsi que 100 µg/ml de kanamycine. Les explants foliaires sont prélevés sur des plants en serre ou in vitro et transformés selon la technique des disques foliaires(Science 1985,Vol 227, p.1229-1231) en trois étapes successives:la première comprend l'induction des pousses sur un milieu MS additionné de 30g/l de saccharose contenant 0.05mg/l d'acide naphtylacétique (ANA) et 2 mg/l de benzylaminopurine (BAP) pendant 15 jours. Les pousses formées au cours de cette étape sont ensuite développées par culture sur un milieu MS additionné de 30 g/l de saccharose mais ne contenant pas d'hormone, pendant 10 jours. Puis on prélève des pousses développées et on les cultive sur un milieu d'enracinement MS à teneur moitié en sels, vitamines et sucres et ne contenant pas d'hormone. Au bout d'environ 15 jours, les pousses enracinées sont passées en terre. Les plantes obtenues sont appellées Co 17.

Au sortir de l'in-vitro, les plantules de tabac transformées ont été acclimatées à la serre (60% d'humidité relative; température: 20°C la nuit et 23°C la jour) pendant cinq semaines puis traitées au 4-[4-CF3-2-(méthylsulfonyl) benzoyl]-5-cyclopropyl isoxazole.

Le tabac témoin, non transformé et traité au 4-[4-CF3-2-(méthylsulfonyl)benzoyl]-5-cyclopropyl isoxazole à des doses allant de 50 à 400 g/ha, développe en environ 72 heures des chloroses, qui s'intensifient pour évoluer vers des nécroses très prononcées en une semaine (couvrant environ 80% des feuilles terminales).

Après transformation ce même tabac, qui surexprime l'HPPD de *P. fluorescens*, est très bien protégé contre un traitement au 4-[4-CF3-2-(méthylsulfonyl) benzoyl]-5-cyclopropyl isoxazole à la dose de 400 g/ha.

Si l'enzyme surexprimée est dans le chloroplaste, c'est à dire si la transformation a été faite avec le gène porté par le vecteur pRP V, alors la plante est parfaitement protégée, ne présente aucun symptôme.

Exemple 4: Transformation du tabac industriel PBD6. avec gène EPSPS pour ⇒ construction 173

Isolement d'un ADNc codant pour une EPSPS de maïs:

Les différentes étapes, qui ont conduit à l'obtention de l'ADNc d'EPSPS de maïs, qui a servi de substrat à l'introduction des deux mutations, sont décrites ci-dessous. Toutes les opérations décrites ci-dessous sont données à titre d'exemples et correspondent à un choix, effectué parmi les différentes méthodes disponibles pour parvenir au même résultat. Ce choix n'a aucune incidence sur la qualité du résultat et par conséquent, toute méthode adaptée peut être utilisée par l'homme de l'art pour parvenir au même résultat. La plupart des méthodes d'ingénierie des fragments d'ADN sont décrites dans "Current Protocols in

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Molecular Biology" Volumes 1 et 2, Ausubel F.M. et al , publiés par Greene Publishing Associates et Wiley -Interscience (1989)(Par la suite, les références à des protocoles décrits dans cet ouvrage seront notées "réf. CPMB"). Les opérations concernant l'ADN, qui ont été effectuées selon les protocoles décrits dans cet ouvrage sont, en particulier les suivantes: ligation de fragments d'ADN, traitements par l'ADN polymérase de Klénow et la T4 ADN polymérase, préparation d'ADN de plasmides et de bactériophages λ soit en minipréparation soit en maxi préparation, analyses d'ADN et d'ARN respectivement selon les techniques de Southern et Northern. D'autres méthodes décrites dans cet ouvrage ont été suivies, et seules les modifications ou ajouts significatifs à ces protocoles ont été décrits ci-dessous.

A1. Obtention d'un fragment d'EPSPS d' ***Arabidopsis thaliana***

a) deux oligonucleotides 20-mers de séquences respectives:

5'- GCTCTGCTCATGTCTGCTCC -3'

5'- GCCCGCCCTTGACAAAGAAA- 3'

ont été synthétisés à partir de la séquence d'un gène d'EPSPS d'*Arabidopsis thaliana* (Klee H.J. et al. (1987) Mol. Gen. Genet., 210, 437-442). Ces deux oligonucleotides sont respectivement en position 1523 à 1543 et 1737 à 1717 de la séquence publiée et en orientation convergente.

b) L'ADN total d'*Arabidopsis thaliana (var. columbia)* a été obtenu chez Clontech (référence catalogue: 6970-1)

c) On mélange 50 nanogrammes(ng) d'ADN avec 300ng de chacun des oligonucleotides et soumis à 35 cycles d'amplification avec un appareil Perkin-Elmer 9600, dans les conditions de milieu standard pour l'amplification préconisées par le fournisseur. Le fragment de 204 pb résultant constitue le fragment d'EPSPS d' *Arabidopsis thaliana*.

2. Construction d'une bibliothèque d'un ADNc à partir d'une ligne cellulaire de maïs BMS .

a) On broye 5 g de cellules filtrées dans l'azote liquide et les acides nucléiques totaux extraits selon la méthode décrite par Shure et al. avec les modifications suivantes:

- le pH du tampon de lyse est ajusté à PH = 9,0;

-après la précipitation par l'isopropanol, le culot est repris dans l'eau et après dissolution, ajusté à 2,5 M LiCl. Après incubation pendant 12 h à °C, le culot de la centrifugation d 15 min. à 30000g à 4°C est resolubilisé. L'étape de précipitation par LiCl est alors répétée. Le culot resolubilisé constitue la fraction ARN des acides nucléiques totaux.

b) La fraction ARN-polyA+ de la fraction ARN est obtenue par chromatographie sur colonne oligo-dT cellulose telle que décrite dans "Current Protocols in Molecular Biology" .

c) Synthèse d'ADNc double brin à extrémité synthétique EcoRI: elle est réalisée en suivant le protocole du fournisseur des différents réactifs nécessaires à cette synthèse sou forme d'un kit: le "copy kit" de la société In Vitrogen.

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Deux oligonucleotides simples brins et partiellement complémentaires de séquences respectives:

5'- AATTCCCGGG -3'

5'- CCCGGG- 3' (ce dernier étant phosphorylé)

sont ligués avec les ADNc double brin à extrémités franches.

Cette ligation des adaptateurs résulte en la création de sites Sma I accolés aux ADNc double brin et EcoRI sous forme cohésive à chaque extrémité des ADNc double brin.

d) Création de la bibliothèque:

Les ADNc présentant à leurs extrémités les sites artificiels cohésifs EcoRI sont ligués avec le ADNc du bactériophage λgt10 coupé par EcoRI et déphosphorylé selon le protocole du fournisseur New England Biolabs.

Une aliquote de la réaction de ligation a été encapsidée *in vitro* avec des extraits d'encapsidation: Gigapack Gold selon les instructions du fournisseur, cette librairie a été titrée en utilisant la bactérie *E.coli* C600*hfl*. la librairie ainsi obtenue est amplifiée et stockée selon les instructions du même fournisseur et constitue la librairie de ADNc de suspension cellulaire de maïs BMS.

3. Criblage de la bibliothèque de ADNc de suspension cellulaire de maïs BMS avec la sonde EPSPS d'*Arabidopsis thaliana:*

Le protocole suivi est celui de "Current Protocols in Molecular Biology" Volumes 1 et 2, Ausubel F.M. et al , publiés par Greene Publishing Associates et S (1989)(CPMB). En bref, environ 10^6 phages recombinants sont étalés sur boîte LB à une densité moyenne de 100 phages /cm^2. Les plages de lyses sont répliqués en doubles sur membrane Hybond N d'Amersham.

h) L'ADN a été fixé sur les filtres par traitement UV 1600kJ (Stratalinker de Stratagene). Les filtres iont été préhydridés dans: 6xSSC/0,1%SDS/0,25 lait écrémé pendant 2h à 65°C. La sonde EPSPS d'*Arabidopsis thaliana* a été marquée au ^{32}P-dCTP par "random-priming" selon les instructions du fournisseur (Kit Ready to Go de Pharmacia). L'activité spécifique obtenue est de l'ordre de 10^8 cpm par µg de fragment. Après dénaturation pendant 5 min à 100°C, la sonde est ajoutée dans le milieu de préhybridation et l'hybridation est poursuivie pendant 14 heures à 55°C. Les filtres sont fluorographiés 48h à -80°C avec un film Kodak XAR5 et des écrans renforçateurs Hyperscreen RPN d'Amersham. L'alignement des spots positifs sur le filtre avec les boîtes d'où ils sont issus permet de prélever, sur la boîte, des zones correspondant aux phages présentant une réponse d'hybridation positive avec la sonde EPSPS d'*Arabidopsis thaliana.* Cette étape d'étalement, transfert, hybridation, récupération est répétée jusqu'à ce que tous les spots de la boîte des phages successivement purifiés se révèlent positifs à 100% en hybridation. Une plage de lyse par phage indépendant est alors prélevée dans du milieu λ diluant (Tris-Cl pH= 7,5; MgSO4

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10mM; NaCl 0,1M; gélatine 0,1%), ces phages en solution constituant les clones positifs de l' EPSP de la suspension cellulaire de maïs BMS.

4. Préparation et analyse de l'ADN des clones d'EPSPS de la suspension cellulaire de maïs BMS.

On ajoute environ 5.10^8 phages à 20 ml de bactéries C600hfl à 2 OD 600nm/ml et incubés 15 minutes à 37°C. Cette suspension est alors diluée dans 200ml de milieu de croissance des bactéries dans un Erlen de 1 l et agitée dans un agitateur rotatif à 250 rpm. La lyse est constatée par clarification du milieu, correspondant à l lyse des bactéries turbides et se produit après environ 4 h d'agitation. Ce surnageant est alors traité comme décrit dans "Current Protocols in Molecular Biology". L'ADN obtenu correspond aux clones d'EPSP de la suspension cellulaire de maïs BMS.

Un à deux µg de cet ADN sont coupés par EcoRI et séparés sur gel d'agarose LGTA/TBE (réf. CPMB) à 0,8%. Une dernière vérification consiste à s'assurer que l' ADN purifié présente bien un signal d'hybridation avec la sonde EPSPS d'*Arabidopsis thaliana*. Après l'électrophorèse, les fragments d'ADN sont transférés sur membrane Hybond N d'Amersham selon le protocole de Southern décrit dans "Current Protocols in Molecular Biology". Le filtre est hybridé avec la sonde EPSPS d'*Arabidopsis thaliana* selon les conditions décrites au paragraphe 3 ci-dessus. Le clone présentant un signal d'hybridation avec la sonde EPSPS d'*Arabidopsis thaliana* et contenant le plus long fragment EcoRI a une taille estimée sur gel à environ 1,7kpb.

5. Obtention du clone pRPA-ML-711:

Dix µg de l'ADN du clone phagique contenant l'insert de 1,7kpb sont digérés par EcoRI et séparés sur gel d'agarose LGTA/TBE (réf. CPMB) à 0,8%. Le fragment de gel contenant l'insert de 1,7kpb est excisé du gel par coloration BET et le fragment est traité à la β-agarase selon le protocole du fournisseur New a Biolabs. L'ADN purifié du fragment de 1,7kpb est ligué à 12°C pendant 14h avec l'ADN du plasmide pUC 19 (New England Biolabs) coupé par EcoRI selon le protocole de ligation décrit dans "Current Protocols in Molecular Biology". Deux µl du mélange de ligation ci-dessus sont utilisés pour la transformation d'une aliquote d'E.coli DH10B électro compétentes; la transformation se fait par électroporation en utilisant les conditions suivantes: le mélange de bactéries compétentes et de milieu de ligation est introduit dans une cuvette d'électroporation d'épaisseur 0,2 cm (Biorad) préalablement refroidie à 0°C. Les conditions physiques de l'électroporation utilisant un électroporateur de marque Biorad sont 2500 Volts, 25 µFarad et 200 Ω. Dans ces conditions, le temps de décharge moyen de condensateur est de l'ordre de 4,2 millisecondes. Les bactéries sont alors reprises dans 1 ml de milieu SOC (réf. CPMB) et agitées pendant 1 heure à 200 rpm sur un agitateur rotatif dans des tubes Corning de 15 ml. Après étalement sur milieu LB/agar supplémenté à 100 µg/ml de carbéniciline, les mini-préparations des clones bactériens ayant poussé après une nuit à 37 °C est réalisée selon le protocole décrit

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dans "Current Protocols in Molecular Biology". Après digestion par EcoRI de l'ADN et séparation en électrophorèse sur gel d'agarose LGTA/TBE (réf. CPMB) à 0.8%, les clones présentant un insert de 1,7kpb sont conservés. Une dernière vérification consiste à s'assurer que l' ADN purifié présente bien un signal d'hybridation avec la sonde EPSPS d'*Arabidopsis thaliana*. Après l'électrophorèse, les fragments d'ADN sont transférés sur membrane Hybond N d'Amersham selon le protocole de Southern décrit dans "Current Protocols in Molecular Biology". Le filtre est hybridé avec la sonde EPSPS d'*Arabidopsis thaliana* selon les conditions décrites au paragraphe 3 ci-dessus. Le clone plasmidique présentant un insert de 1,7kpb et hybridant avec la sondé EPSPS d'*Arabidopsis thaliana* a été préparé à plus grande échelle et l'ADN résultant de la lyse des bactéries purifié sur gradient de CsCl ainsi que décrit dans "Current Protocols in Molecular Biology". L'ADN purifié a été partiellement séquencé avec un kit Pharmacia en suivant les instructions du fournisseur et en utilisant comme amorces, les amorces universelles de M13 directes et inverses commandées chez le même fournisseur. La séquence partielle réalisée couvre environ 0,5 kpb. La séquence dérivée en acides aminés dans la région de la protéine mature (environ 50 résidus acides aminés) présente une identité de 100% avec la séquence aminée correspondante de l'EPSPS mature de maïs décrite dans le brevet américain USP 4 971 908). Ce clone correspondant à un fragment EcoRI de 1,7kpb de l'ADN de l' EPSP de la suspension cellulaire de maïs BMS a été nommé pRPA-ML-711. La séquence complète de ce clone a été réalisée sur les deux brins en utilisant le protocole du kit Pharmacia et en synthétisant des oligonucléotides complémentaires et de direction opposée tous les 250 pb environ. La séquence complète de ce clone de 1713 pb obtenue est présentée par SEQ ID N° 2.

6. Obtention du clone pRPA-ML-715:

L'analyse de la séquence du clone pRPA-ML-711 et en particulier la comparaison de la séquence d' acides aminés dérivés avec celle de maïs montre une extension de séquence de 92 pb en amont du codon GCG codant pour l'Alanine NH2-terminale de la partie mature de l'EPSPS de maïs (brevet américain USP 4 971 908). De même une extension de 288 pb en aval du codon AAT codant pour l'asparagine COOH-terminale de la partie mature de l'EPSPS de maïs (brevet américain USP 4 971 908) est observée. Ces deux parties pourraient correspondre, pour l'extension NH2-terminale à une portion de la séquence d'un peptide de transit pour la localisation plastidiale et pour l'extension COOH-terminale à la région 3' non traduite de l'ADNc.

Afin d'obtenir un ADNc codant pour la partie mature de l'ADNc de l'EPSPS de maïs, telle que décrite dans l' USP 4 971 908, les opérations suivantes ont été réalisées:

a) Elimination de la région 3' non traduite: construction de pRPA-ML-712:

Le clone pRPA-ML-711 a été coupé par l'enzyme de restriction AseI et les extrémités résultant de cette coupure rendues franches par traitement avec le fragment de Klenow de l'ADN polymérase I selon le protocole décrit dans CPMB. Une coupure par l'enzyme de

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restriction SacII a ensuite été effectuée. L'ADN résultant de ces opérations a été séparé par électrophorèse sur gel d'agarose LGTA/TBE (réf. CPMB) 1%.

Le fragment de gel contenant l'insert "AseI-extrémités franches/SacII" de 0.4 kpb a été excisé du gel et purifié selon le protocole décrit au paragraphe 5 ci-dessus. L'ADN du clone pRPA-ML-711 a été coupé par l'enzyme de restriction HindIII située dans le polylinker du vecteur de clonage pUC19 et les extrémités résultant de cette coupure ont été rendues franches par traitement avec le fragment de Klenow de l'ADN polymérase I. Une coupure par l'enzyme de restriction SacII a ensuite été effectuée. L'ADN résultant de ces manipulations a été séparé par électrophorèse sur gel d'agarose LGTA/TBE (réf. CPMB) 0,7%.

Le fragment de gel contenant l'insert HindIII-extrémités franches/SacII de environ 3,7kpb a été excisé du gel et purifié selon le protocole décrit au paragraphe 5 ci-dessus.

Les deux inserts ont été ligués, et 2 µl du mélange de ligation ont servi à transformer E. *coli* DH1OB ainsi que décrit plus haut au paragraphe 5.

On analyse le contenu en ADN plasmidique de différents clones selon la procédure décrite pour pRPA-ML-711. Un des clones plasmidique retenu contient un insert EcoRI-HindIII de 1,45 kpb environ. La séquence des extrémités terminales de ce clone révèle que l'extrémité 5' de l'insert correspond exactement à l'extrémité correspondante de pRPA-ML-711 et que l'extrémité 3' terminale présente la séquence suivante:

" 5'-...AATTAAGCTCTAGAGTCGACCTGCAGGCATGCAAGCTT-3' ".

La séquence soulignée correspond au codon de l'acide aminé COOH-terminal asparagine, le codon suivant correspondant au codon stop de la traduction. Les nucléotides en aval correspondent à des éléments de séquence du polylinker de pUCI9. Ce clone comprenant la séquence de pRPAML-711 jusqu'au site de terminaison de la traduction de l'EPSPS mature de maïs et suivie de séquences du polylinker de pUC 19 jusqu'au site HindIII a été nommé pRPA-ML-712.

b) Modification de l'extrémité 5' de pRPA-ML-712: construction de pRPA-ML-715

Le clone pRPA-ML-712 a été coupé par les enzymes de restrictions PstI et HindIII. L'ADN résultant de ces manipulations a été séparé par électrophorèse sur gel d'agarose LGTA/TBE (réf. CPMB) 0,8%. Le fragment de gel contenant l'insert PstI/EcoRI de 1,3 kpb a été excisé du gel et purifié selon le protocole décrit au paragraphe 5 ci-dessus. Cet insert a été mis en ligation en présence de quantité équimoléculaire de chacun des deux oligonucléotides partiellement complémentaires, de séquence:

Oligo1: 5'-GAGCCGAGCTCCATGGCCGGCGCCGAGGAGATCGTGCTGCA-3'

Oligo 2: 5'-GCACGATCTCCTCGGCGCCGGCCATGGAGCTCGGCTC-3'

ainsi qu'en présence d'ADN du plasmide pUCI9 digéré par les enzymes de restrictions BamHI et HindIII.

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Deux µl du mélange de ligation ont servi à transformer *E. coli* DHlOB ainsi que décrit plus haut au paragraphe 5. Après analyse du contenu en ADN plasmidique de différents clones selon la procédure décrite ci-dessus au paragraphe 5, un des clones présentant un insert d'environ 1,3 kpb a été conservé pour analyses ultérieures. La séquence de l'extrémité 5' terminale du clone retenu révèle que la séquence ADN dans cette région est la suivante: séquence du polylinker de pUC19 des sites EcoRI à BamHI, suivi de la séquence des oligonucléotides utilisés lors du clonage, suivi du reste de la séquence présente dans pRPAML-712. Ce clone a été nommé pRPA-ML-713. Ce clone présente un codon methionine ATG inclus dans un site NcoI en amont du codon Alanine N-terminal de l'EPSPSynthase mature. De plus, les codons alanine et glycine de l'extrémité N-terminale ont été conservées, mais modifiées sur la troisième base variable : GCGGGT initial donne GCCGGC modifié.

Le clone pRPA-ML-713 a été coupé par l'enzyme de restriction HindIII et les extrémités de cette coupure rendues franches par traitement avec le fragment de Klenow de la ADN polymérase I. Une coupure par l'enzyme de restriction SacI a ensuite été effectuée. L'ADN résultant de ces manipulations a été séparé par électrophorèse sur gel d'agarose LGTA/TBE (réf. CPMB) 0,8%. Le fragment de gel contenant l'insert "HindIII-extrémités franches/SacI" de 1,3 kpb a été excisé du gel et purifié selon le protocole décrit au paragraphe 5 ci-dessus. Cet insert a été mis en ligation en présence d'ADN du plasmide pUC19 digéré par l'enzyme de restriction XbaI et les extrémités de cette coupure rendues franches par traitement avec le fragment de Klenow de l'ADN polymérase I. Une coupure par l'enzyme de restriction SacI a ensuite été effectuée. Deux µl du mélange de ligation ont servi à transformer *E. coli* DHlOB ainsi que décrit plus haut au paragraphe 5. Après analyse du contenu en ADN plasmidique de différents clones selon la procédure décrite ci-dessus au paragraphe 5, un des clones présentant un insert d'environ 1,3 kpb a été conservé pour analyses ultérieures. La séquence des extrémités terminales du clone retenu révèle que la séquence ADN est la suivante: séquence du polylinker de pUCl9 des sites EcoRI à SacI, suivie de la séquence des oligonucléotides utilisés lors du clonage délétée des 4 pb GATCC de l'oligonucléotide 1 décrit ci-dessus, suivi du reste de la séquence présente dans pRPA-ML-712 jusqu'au site HindIII et séquence du polylinker de pUCl9 de XbaI à HindIII. Ce clone a été nommé pRPA-ML-715.

7) Obtention d'un ADNc codant pour une EPSPS de maïs mutée

Toutes les étapes de mutagénèse ont été réalisées avec le U.S.E. mutagenesis kit de Pharmacia en suivant les instructions du fournisseur. Le principe de ce système de mutagénèse est le suivant: l'ADN plasmidique est dénaturé par la chaleur et réassocié en présence d'un excès molaire d'une part de l'oligonucléotide de mutagénèse, et d'autre part d'un oligonucléotide permettant d'éliminer un site d'enzyme de restriction unique présent

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dans le polylinker. Après l'étape de réassociation, la synthèse du brin complémentaire est réalisée par l'action de la T4 ADN polymérase en présence de T4 ADN ligase et de protéine du gène 32 dans un tampon approprié fourni. Le produit de synthèse est incubé en présence de l'enzyme de restriction, dont le site est supposé avoir disparu par mutagénèse. La souche d'*E. coli* présentant, en particulier, la mutation mutS est utilisée comme hôte pour la transformation de cet ADN. Après croissance en milieu liquide, l'ADN plasmidique total est préparé, incubé en présence de l'enzyme de restriction utilisée précédemment. Après ces traitements, la souche d'*E. coli* DH1OB est utilisée comme hôte pour la transformation. L'ADN plasmidique des clones isolés est préparé et la présence de la mutation introduite vérifiée par séquençage.

A)- modifications de sites ou de séquence sans incidence a priori sur le caractère de résistance de l'EPSPS de maïs aux produits inhibiteurs compétitifs de l'activité EPSP synthase: élimination d'un site NcoI interne de pRPA-ML-715.

La séquence de pRPA-ML-715 est numérotée arbitrairement en plaçant la première base du codon Alanine N-terminal GCC en position 1. Cette séquence présente un site NcoI en position 1217. L'oligonucléotide de modification du site présente la séquence :

5'-CCACAGGATGGCGATGGCCTTCTCC-3'.

Après séquençage selon les références données ci-dessus, la séquence lue après mutagénèse correspond à celle de l'oligonucléotide utilisé. Le site NcoI a bien été éliminé et la traduction en acides aminés dans cette région conserve la séquence initiale présente sur pRPA-ML-715.

Ce clone a été nommé pRPA-ML-716.

La séquence de 1340 bp de ce clone est présentée SEQ ID N° 3 et SEQ ID N° 4.

B) modifications de séquence permettant l'augmentation du caractère de résistance de l'EPSPS de maïs aux produits inhibiteurs compétitifs de l'activité EPSP synthase.

Les oligonucléotides suivants ont été utilisés :

a) mutation Thr 102 ➡ Ile.

5'-GAATGCTGGAATCGCAATGCGGCCATTGACAGC-3'

b) mutation Pro 106 ➡ Ser.

5'-GAATGCTGGAACTGCAATGCGGTCCTTGACAGC-3'

c) mutations Gly 101 ➡ Ala et Thr 102 ➡ Ile.

5'-CTTGGGGAATGCTGCCATCGCAATGCGGCCATTG-3'

d) mutations Thr 102 ➡ Ile et Pro 106 ➡ Ser.

5'-GGGGAATGCTGGAATCGCAATGCGGTCCTTGACAGC-3'

Après séquençage, la séquence lue après mutagénèse sur les trois fragments mutés est identique à la séquence de l'ADN parental pRPA-ML-716 à l'exception de la région mutagénéisée qui correspond à celle des oligonucléotides de mutagénèse utilisés. Ces clones

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ont été nommés : pRPA-ML-717 pour la mutation Thr 102 ➡ Ile, pRPA-ML-718 pour la mutation Pro 106 ➡ Ser, pRPA-ML-719 pour les mutations Gly 101 ➡ Ala et Thr 102 ➡ Ile et pRPA-ML-720 pour les mutations Thr 102 ➡ Ile et Pro 106 ➡ Ser.

La séquence de 1340 bp de pRPA-ML-720 est présentée SEQ ID N° 5 et SEQ ID N° 6.

L'insert *NcoI-HindIII* de 1395 pb est à la base de toutes les constructions utilisées pour la transformation des plantes pour l'introduction de la résistance aux herbicides inhibiteurs compétitifs de l'EPSPS et en particulier la résistance au glyphosate. Cet insert sera nommé dans la suite des descriptions "le double mutant de l'EPSPS de maïs".

B Tolérance au glyphosate des différents mutants in vitro.

2.a: Extraction de l'EPSP synthase.

Les différents gènes d'EPSP synthases sont introduits sous forme d'une cassette NcoI-HindIII dans le vecteur plasmidique pTrc99a (Pharmacia, ref : 27-5007-01) coupé par NcoI et HindIII. Les *E. coli* DH10B recombinantes surexprimant les différents EPSP synthases sont soniquées dans 40 ml de tampon par 10 g de cellules culottées et lavées avec ce même tampon (tris HCl 200 mM pH 7,8, mercaptoethanol 50 mM, EDTA 5 mM et PMSF 1 mM), auxquels on ajoute 1 g de polyvinylpyrrolidone. La suspension est agitée pendant 15 minutes à 4°C, puis centrifugée 20 minutes à 27000g et 4°C.

Le surnageant est additionné de sulfate d'ammonium pour amener la solution à 40% de la saturation en sulfate d'ammonium. Le mélange est centrifugé 20 minutes à 27000g et 4°C. Le nouveau surnageant est additionné de sulfate d'ammonium pour amener la solution à 70% de la saturation en sulfate d'ammonium. Le mélange est centrifugé 30 minutes à 27000g et 4°C. L'EPSP synthase, présente dans ce culot protéique, est reprise dans 1 ml de tampon (tris HCl 20 mM pH 7.8 et mercaptoethanol 50 mM). Cette solution est dialysée une nuit contre deux litres de ce même tampon à 4°C.

2.b: Activité enzymatique.

L'activité de chaque enzyme ainsi que sa résistance au glyphosate est mesurée in vitro sur 10 minutes à 37°C dans le mélange réactionnel suivant: acide maléique 100 mM pH 5,6, phosphoénol pyruvate 1 mM, shikimate-3-phosphate 3 mM (préparé selon Knowles P.F. et Sprinson D.B. 1970. Methods in Enzymol 17A, 351-352 à partir de *Aerobacter aerogenes* strain ATCC 25597) et fluorure de potassium 10 mM. L'extrait enzymatique est ajouté au dernier moment après l'addition de glyphosate dont la concentration finale varie de 0 à 20 mM.

L'activité est mesurée par dosage du phosphate libéré selon la technique de Tausky H.A. et Shorr E. 1953. J. Biol. Chem. 202, 675-685.

Dans ces conditions, l'enzyme sauvage (WT) est inhibée à 85% dès la concentration de 0,12 mM de glyphosate. A cette concentration, l'enzyme mutante connue Ser106 n'est inhibée

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qu'à 50% et les trois autres mutants Ile102, Ile102/Ser106, Ala101/Ile102 ne sont pas ou peu inhibées.

Il faut multiplier la concentration de glyphosate par dix, soit 1,2 mM, pour inhiber l'enzyme mutante Ile102 à 50%, les mutants Ile102/Ser106, Ala/Ile et Ala n'étant toujours pas inhibés.

Il faut noter que l'activité des mutants Ala/Ile et Ala n'est pas inhibée jusqu'à des concentrations de 10 mM de glyphosate, et que celle du mutant Ile102/Ser106 n'est pas réduite même si la concentration en glyphosate est multipliée par 2, soit 20 mM.

C

Résistance des plantes de tabac transformés.

0-1 Constructions des plasmides:

pRPA-RD-124: Addition d'un signal de polyadénylation "nos" à pRPA-ML-720, obtenu précédemment, avec création d'une cassette de clonage contenant le gène d'EPSPS double mutant de maïs (Thr 102 → Ile et Pro 106 → Ser). pRPA-ML-720 est digéré avec Hind III, traité avec le fragment de Klenow de l'ADN polymérase I d'*E. coli* pour produire une extrémité franche. On effectue une seconde digestion avec Nco I et le fragment EPSPS est purifié. Le gène EPSPS est ensuite ligué avec pRPA-RD-12 purifié (une cassette de clonage contenant le signal de polyadénylation de la nopaline synthase) pour donner pRPA-RD-124. Pour obtenir le vecteur pRPA-RD-12 purifié utile, il a fallu que celui-ci soit préalablement digéré par SalI, traité avec l'ADN polymérase de Klenow, puis digéré une seconde fois avec NcoI.

pRPA-RD-125: Addition d'un peptide de transit optimisé (OTP) à pRPA-RD-124 avec création d'une cassette de clonage contenant le gène d'EPSPS ciblé sur les plasmides. pRPA-RD-7 (demande de brevet européen EP 652 286) est digéré avec Sph I, traité avec la T4 ADN polymérase, puis digéré avec Spe 1 et le fragment OTP est purifié. Ce fragment OTP est cloné dans pRPA-RD-124 qui a été préalablement digérée par NcoI, traité avec l'ADN polymérase de Klenow pour enlever la partie protubérante 3', puis digérée par Spe I. Ce clone est alors séquencé pour assurer la fusion traductionnelle correcte entre le OTP et le gène d'EPSPS. On obtient alors pRPA-RD-125.

pRPA-RD-159: Addition du promoteur double d'histone d'*arabidopsis* H4A748 (demande de brevet EP 507 698) à pRPA-RD-125 avec création d'une cassette pour expression dans les plantes pour l'expression du gène "OTP- gène d'EPSPS double mutant" dans les tissus de dicotylédones. pRPA-RD-132 (une cassette contenant le promoteur double H4A748 (demande de brevet EP 507 698)) est digérée avec Nco I et Sac I. Le fragment purifié du promoteur est ensuite cloné dans qui a été digéré avec Eco I et Sac I.

pRPA-RD-173: Addition du gène "promoteur H4A748-OTP-gène d'EPSPS double mutant" de pRPA-RD-159 dans plasmide pRPA-BL-150A (demande de brevet européen 508 909) avec création d'un vecteur de transformation *Agrobacterium tumefaciens*. pRPA-

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RD-159 est digéré avec Not I et traité avec la polymérase de Klenow. Ce fragment est ensuite cloné dans pRPA-BL-150A avec Sma I.

1-1- Transformation.

Le vecteur pRPA-RD-173 est introduit dans la souche *d'Agrobacterium tumefaciens* EHA101 (Hood et al.,1987) porteuse du cosmide pTVK291 (Komari et al.,1986). La technique de transformation est basée sur la procédure de Horsh et al.(1985).

1-2- Régénération.

La régénération du tabac PBD6 (provenance SEITA France) à partir d'explants foliaires est réalisée sur un milieu de base Murashige et Skoog (MS) comprenant 30g/l de saccharose ainsi que 200 µg/ml de kanamycine. Les explants foliaires sont prélevés sur des plantes cultivées en serre ou *in vitro* et transformées selon la technique des disques foliaires (Science,1985,Vol 227,p.1229-1231) en trois étapes successives: la première comprend l'induction des pousses sur un milieu additionné de 30g/l de saccharose contenant 0,05 mg/l d'acide naphtylacétique (ANA) et 2mg/l de benzylaminopurine (BAP) pendant 15 jours. Les pousses formées au cours de cette étape sont ensuite développées pendant 10 jours par culture sur un milieu MS additionné de 30g/l de saccharose mais ne contenant pas d'hormone. Puis on prélève des pousses développées et on les cultive sur un milieu d'enracinement MS à teneur moitié en sels, vitamines et sucre et ne contenant pas d'hormone. Au bout d'environ 15 jours, les pousses enracinées sont passées en terre.

1-3- Résistance au glyphosate.

Vingt plantes transformées ont été régénérées et passées en serre pour la construction pRPA-RD-173. Ces plantes ont été traitées en serre au stade 5 feuilles avec une suspension acqueuse de RoundUp correspondant à 0,8kg de matière active glyphosate par hectare.

Les résultats correspondent à l'observation d'indices de phytotoxicité relevés 3 semaine après traitement. Dans ces conditions, on constate que les plantes transformées par la construction pRPA-RD-173 présentent une très bonne tolérance alors que les plantes témoins non transformées sont complètement détruites.

Ces résultats montrent clairement l'amélioration apportée par l'utilisation d'un gène chimère selon l'invention pour un même gène codant pour la tolérance au glyphosate.

Exemple 5: Transformation du tabac industriel PBD6, avec gène de la nitrilase (pour ⇒ construction 238:

Ce tabac est obtenu selon l'enseignement de la demande européenne n° 0 337 899 page 6 ligne 50 et suivantes à partir de la construction 238, qui est celle décrite sous le nom de pRPA-BL 238.

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Exemple 6: Croisement par pollinisation

On procède par pollinisation en serre au croisement respectivement des lignées Co 17, 173 et 238 :

- Co 17 avec 238 pour obtenir des plantes de tabac PBD6 à tester sur la double tolérance à l'isoxaflutole et au bromoxynil (" plantes HPPD + OXY") et

- Co 17 avec 173 pour obtenir des plantes de tabac PBD6 à tester sur la double tolérance à l'isoxaflutole et au glyphosate ("plantes HPPD + EPSPS").

Les trois lignées sont homozygotes vis à vis du gène concerné: en conséquence la descendance est hemizygote pour chacun des deux gènes introduits par croisement.

Les plantes croisées sont obtenues au bout de six semaines.

Exemple 7: Mesure de la tolérance du tabac en traitement de postlevée avec l'isoxaflutole et de postlevée avec le bromoxynil ou le glyphosate.

Dans cet essai, chaque test est effectué sur un échantillon de 10 plantes, 10 plantes étant gardées non traitées.

Tous les traitements sont effectués par pulvérisation à raison de 500l de bouillie par hectare.

Pour le traitement en postlevée, on fait un semis puis on repique les plantes en godets de 9cm × 9cm.

Les traitements de post levée sont faits à un stade bien développé (3-4 feuilles)Des lots de plantes respectivement sauvage et génétiquement transformées obtenues ci-dessus sont répartis en plusieurs parts, avec:

a) un lot non traité,

b) d'autres lots qui sont traités respectivement avec un herbicide seul,

- de l'isoxaflutole en post levée, à deux doses (respectivement 200 et 400 g/ha) ,
- du bromoxynil en post levée à deux doses (respectivement 400 et 800 g/ha),,
- du glyphosate en post levée à deux doses (respectivement 800 et 1200 g/ha),

c) d'autres lots qui sont traités respectivement avec deux herbicides, en post levée, en mélange extemporané:

- de l'isoxaflutole et du bromoxynil à deux doses (respectivement 200/400 et 400/800 g/ha)
- de l'isoxaflutole et du glyphosate à deux doses (respectivement 200/800 et 400/1200 g/ha).

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Les traitements sont effectués avec les formulations suivantes: isoxaflutole à 75%, le bromoxynil (produit commercial PARDNER) sous forme octanoate en concentré émulsionnable à 225g /l et le glyphosate (Round-UP)

Dans ces conditions, on observe 17 jours après le traitement les phytotoxicités suivantes, exprimées en pourcentage de destruction indiquées dans le tableau suivant, ainsi que le nombre de plantes par lot et les doses d'herbicide(s) exprimées en gramme de matière active par hectare:

Traitement de postlevée avec l'isoxaflutole
et de postlevée avec le bromoxynil ou le glyphosate

Herbicide en g/l		Plantes avec gène de tolérance					
		*	HPPD + OXY	*	HPPD + EPSPS	*	SANS = Sauvage
Contrôles		10		10		10	
isoxaflutole	200	20	4%	20	2%	10	75%
seul	400	20	5%	20	3%		85%
bromoxynil	400	10	3%			10	0%
seul	800	10	0%			10	0%
Glyphosate	800			20	0%	10	100%
seul	1200			20	0%	10	100%
Isoxaflutole	200	20	20%			10	100%
+ bromoxynil	400						
Isoxaflutole	400	20	30%			10	100%
+ bromoxynil	800						
Isoxaflutole	200			40	5%	10	100%
+ glyphosate	800						
Isoxaflutole	400			40	10%	10	100%
+ glyphosate	1200						

* nombre de plants

Exemple 8

Dans le but d'étudier si le gène de l'HPPD de *Pseudomonas fluorescens* peut être utilisé comme gène marqueur au cours du cycle "transformation - régénération" d'une espèce végétale, le tabac a été transformé avec le gène chimère composé du gène de l'HPPD et du gène EPSPS doublement muté de résistance au glyphosate et des plantes transformées

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résistantes à la fois à l'isoxaflutole et au glyphosate ont été obtenues après sélection sur isoxaflutole.

Matériel et méthodes et résultats

Le gène chimérique pRP 2012 décrit ci-dessous est transféré dans du tabac industriel PBD6 selon les procédures de transformation et de régénération déjà décrites dans la demande européenne EP n° 0 508 909.

Le gène chimère du vecteur pRP 2012 a la structure A-B suivante, dans laquelle:

A est:

Promoteur double histone	TEV	OTP	Région codante de l'HPPD	Terminateur nos

et B est :

Promoteur double histone	TEV	OTP	Région codante de l'EPSPS	Terminateur nos

comme celui utilisé dans le vecteur pRPA-RD-173

Le gène chimère pRP 2012 est introduit dans le tabac.

1)Transformation:

Le vecteur est introduit dans la souche non oncogène d'*Agrobacterium* EHA 101(Hood et al,1987) porteuse du cosmide pTVK 291(Komari et al,1986). La technique de transformation est basée sur la procédure de Horsh et al(1985).

2) Régénération:

La régénération du tabac PBD6 (provenance SEITA France) à partir d'explants foliaires est réalisée sur un milieu de base Murashige et Skoog (MS) comprenant 30g/l de saccharose ainsi que 350 mg/l de cefotaxime et 1 mg/l d'isoxaflutole. Les explants foliaires sont prélevés sur des plants en serre ou in vitro et transformés selon la technique des disques foliaires (Science 1985,Vol 227,p.1229-1231) en trois étapes successives: la première comprend l'induction des pousses sur un milieu MS additionné de 30g/l de saccharose contenant 0.05mg/l d'acide naphtylacétique (ANA) et 2 mg/l de benzylaminopurine (BAP) pendant 15 jours et 1 mg/l d'isoxaflutole. Les pousses vertes formées au cours de cette étape sont ensuite développées par culture sur un milieu MS additionné de 30 g/l de saccharose et 1 mg/l d'isoxaflutole mais ne contenant pas d'hormone, pendant 10 jours. Puis on prélève des

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pousses développées et on les cultive sur un milieu d'enracinement MS à teneur moitié en sels, vitamines et sucres et 1 mg/l d'isoxaflutole et ne contenant pas d'hormone. Au bout d'environ 15 jours, les pousses enracinées sont passées en terre.

Toutes les plantules obtenues selon ce protocole sont analysées par PCR avec des amorces spécifiques de l'HPPD de *P.fluorescens*. Cette analyse PCR a permis de confirmer que toutes les plantules ainsi obtenues ont bien intégré le gène de l'HPPD et qu'elles sont tolérantes à la fois à l'isoxaflutole et au glyphosate, dans les conditions décrites à l'exemple7.

En conclusion, cet essai confirme que le gène de l'HPPD peut être utilisé comme gène marqueur et que, associé à ce gène, l'isoxaflutole peut être un bon agent de sélection.

Exemple 9: Plante avec un gène HPPD et un gène *bar*, résistant à la fois à l'isoxaflutole et à la phosphinothrycine.

1. Construction d'un gène chimère avec une séquence HPPD:

Le plasmide pRPA-RD-1004 représenté à la figure 4 est obtenu par insertion du gène chimère de résistance aux isoxazoles dans le plasmide pUC 19 de 2686 pb, commercialisé par New Englamd Biolabs (Yannish-Perron, C.Viera, J. and Massing, J. (1985) Gene 33, 103-119 et contenant la résistance à l'ampicilline.

Les différents éléments du gène chimère sont, dans le sens de la traduction:

- le promoteur histone H3C4 de maïs de 1020pb décrit dans la demande EP 0 507 698;
- l'intron du gène de l'alcool déshydrogénase 1 de maïs décrit par Sachs M *et al.* Genetics 113: 449-467 (1986) et constitué de 536pb
- le peptide de tansit optimisé (OTP) décrit dans la demande de brevet EP 0 508 909; cet OTP est constitué des 171 pb du peptide de transit de la petite sous unité de la Ribulose 1,5 bisphosphate carboxylase /oxygénase d'*Helianthus annuus* (Waksman G. *et al* 1987. Nucleics acids Res. 15: 7181) suivies des 66 pb de la partie mature de la petite sous unité de la Ribulose 1,5 bisphosphate carboxylase /oxygénase de *Zea mays* (Lebrun *et al* 1987. Nucleics acids Res. 15: 4360) elles mêmes suivies des 150 pb du peptide de transit de la petite sous unité de la Ribulose 1,5 bisphosphate carboxylase/ oxygénase de *Zea mays* (Lebrun *et al* 1987. Nucleics acids Res. 15: 4360); L'ensemble fait donc 387 pb;
- la région codante de l'HPPD de *Pseudomonas fluorescens* décrite ci-dessus;
- le terminateur du gène de la nopaline synthase (nos) (zone de polyadenylation du gène *nos* isolé de pTi 37, 250 pb (Bevan M. et al. Nucleics Acids Res. 11: 369-385);

2. construction d'un gène chimère de tolérance à la phosphinothricine (gène *bar*):

La phosphinothricine acetyl tranferase (PAT) codée par le gène *bar* est une enzyme qui inactive un herbicide, la phosphinothricine (PPT). La PPT inhibe la synthèse de

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glutamine et provoqie une accumulation rapide d'ammoniaque dans les cellus conduisant à leur mort (Tachibana *et al.* 1986) .

Le plasmide utilisé pour introduire la tolérance à la phosphinothricine comme agent de sélection est obtenu par insertion du gène chimère pDM 302 dans le vecteur pSP72 de 2462 pb, commercialisé par Promega Corp. (Genbank/ DDBJ database accession number X65332) et contenant le gène de résistance à l'amplicilline.

Le plasmide pDM 302 de 4700pb a été décrit par Cao, J., *et al.* Plant Cell Report 11: 586-591 (1992).

Les différents éléments de ce plasmide sont:

- le promoteur du gène actine de riz décrit par Mc Elroy D. *et al.* Plant Molecular Biology 15: 257-268 (1990) constitué de 840 pb;
- le premier exon du gène actine de riz constitué de 80 pb;
- le premier intron du gène actine de riz constitué de 450 pb;- la région codante du gène *bar* de 600 pb excisée du plasmide pIJ41404 décrit par White J. *et al.* Nuc. Acids res. 18: 1862 (1990);
- le terminateur du gène de la nopaline synthase (nos) (zone de polyadenylation du gène *nos* isolé de pTi 37, 250 pb (Bevan M. et al. Nucleics Acids Res. 11: 369-385).

3. transformation:

La technique du bombardement est utilisée pour introduire la construction génétique. Les palsmides sont purifiés sur colonne Qiagen et coprécipités sur particules de tungstène M10 selonle procédé Klein (Nature 327: 70-73, 1987).

Une mixture (?) de particules métalliques et des deux plasmides décrits ci-dessus est ensuite n

bombardées sur des cellules embryogènes de maïs selon le protocole par (???)

4. Régénération et utilisation du gène *bar* comme agent de sélection:

Les cals bombardés sont sélectionnés sur glufosinate jusqu'à l'apparition de setceurs verts. Les cals positifs (?) sont alors convertis en embryons somatiques *(conditions ou référence de la technique?)* puis mis dans les conditions favorisant la germination *(conditions ou référence de la technique?).* Les jeunes plantes son transférées en serre pour la production de graines *(conditions ou référence de la technique?).*

Les analyse moléculaires *(conditions ou référence de la technique?PCR?)* réalisées sur ces plantes montrent que :

- au moins 4 cals sélectionnés sur phosphinothricine ont engendré des plantes révélant la présence du gène de l'HPPD par PCR;

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- au moins 5 cals sélectionnés sur phosphinothricine ont engendré des plantes révélant la présence du gène de l'HPPD par Southern blot;
- au moins 5 cals sélectionnés sur phosphinothricine ont engendré des plantes révélant la présence de la protéine recombinante par Western blot;
- le gène chimère de l'HPPD et la protéine hétérologue sont absents des cals non transformés.

Ces résultats montrent l'efficacité du gène chimère *bar* pour la sélection des cals transformés contenant un autre gène d'intérêt agronomique.

5. Analyse de la descendance des plantes transformées:

Les plantes transformées obtenues ci-dessus ont émis du pollen supposé en partie transgénique, qui a fécondé des ovules d'un maïs sauvage non transgénique. Les graines obtenues sont sélectionnées sur sable après traitement à l'isoxaflutole. Le protocole de sélection est le suivant:

800ml de sable de Fontainebleau sont placés dans une barquette de 15 × 20 cm de côtés. Ces barquettes sont alors arrosées oar de l'eau et maintenant hydratées par apport d'une solution nutritive constituée de 5ml de Quinoligo (La Quinoléine) par litre d'eau. Vingt graines de maïs sont placées sur les barquettes, qui sont alors traitées à l'isoxaflutole par pulvérisation à raison de 100 à 200g de matière active par hectare (300 ou 600 µg de matière active par barquette). Les barquettes sont ensuite placées en culture en serre.

Les résultats obtenus sont rassemblés dans le tableau suivant:

Génotypes	Isoxaflutole (g/ha)	nombre de graines semées	nombre de plantes germées	nombre de plantes mortes	nombre de plantes survivantes
non transgénique	0	20	20	0	20
	100	20	20	20	0
261 2B 459	100	10	10	5	5
	200	10	9	4	5
261 2D2	100	10	9	6	3
	200	10	10	7	3
261 2A2	100	10	5	3	2
	200	10	7	7	0

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Ces résultats montrent l'efficacité du gène de l'HPPD pour la sélection des plantes résistantes de maïs. Ils montrent aussi que la surexpression de l'HPPD de *Pseudomonas* dans les tissus de maïs leur confère la tolérance à l'isoxaflutole.

Les séquences illustrées sont les suivantes:

SEQ ID N° 1

Séquence du gène de l'HPPD de *Pseudomonas fluorescens* A32.

SEQ ID N° 2

Séquence d' ADNc d'EPSPS d'*Arabidopsis thaliana*

SEQ ID N° 3 et 4

séquences respectivement du gène et de la protéine de l'EPSPS de maïs mutée, partie 1340 pb du clone pRPA-ML-716

SEQ ID n° 5 et SEQ ID n° 6

séquences respectivement du gène et de la protéine de l'EPSPS de maïs mutée, partie 1340 pb du clone pRPA-ML-720

Les figures ci-après sont données à titre indicatif pour illustrer l'invention.

La Figure 1 représente la séquence protéique de l'HPPD de *Pseudomonas sp.* strain P.J. 874 et la séquence nucléotidique théorique de la partie codante correspondante; les cinq oligonucléotides choisis pour faire l'amplification d'une partie de cette région codante sont symbolisés par les cinq flèches.

La Figure 2 représente la cartographie du plasmide avec le fragment d'ADN génomique de 7 kb contenant le gène de l'HPPD de *P. fluorescens* A32.

La Figure 3 donne la comparaison des séquences en acides aminés de l' HPPD de *P. fluorescens A32* et de l'HPPD de *Pseudomonas sp* strain P.J. 874 (seuls les acides aminés divergents entre les deux séquences sont indiqués) ainsi que la séquence consensus.

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Liste des séquences

SEQ ID NO: 1:

ATGGCAGATC TATACGAAAA CCCAATGGGC CTGATGGGCT TTGAATTCAT CGAATTCGCG 60

TCGCCGACGC CGGGTACCCT GGAGCCGATC TTCGAGATCA TGGGCTTCAC CAAAGTCGCG 120

ACCCACCGTT CCAAGAACGT GCACCTGTAC CGCCAGGGCG AGATCAACCT GATCCTCAAC 180

AACGAGCCCA ACAGCATCGC CTCCTACTTT GCGGCCGAAC ACGGCCCGTC GGTGTGCGGC 240

ATGGCGTTCC GCGTGAAGGA CTCGCAAAAG GCCTACAACC GCGCCCTGGA ACTCGGCGCC 300

CAGCCGATCC ATATTGACAC CGGGCCGATG GAATTGAACC TGCCGGCGAT CAAGGGCATC 360

GGCGGCGCGC CGTTGTACCT GATCGACCGT TTCGGCGAAG GCAGCTCGAT CTACGACATC 420

GACTTCGTGT ACCTCGAAGG TGTGGAGCGC AATCCGGTCG GTGCAGGTCT CAAAGTCATC 480

GACCACCTGA CCCACAACGT CTATCGCGGC CGCATGGTCT ACTGGGCCAA CTTCTACGAG 540

AAATTGTTCA ACTTCCGTGA AGCGCGTTAC TTCGATATCA AGGGCGAGTA CACCGGCCTG 600

ACTTCCAAGG CCATGAGTGC GCCGGACGGC ATGATCCGCA TCCCGCTGAA CGAAGAGTCG 660

TCCAAGGGCG CGGGGCAGAT CGAAGAGTTC CTGATGCAGT TCAACGGCGA AGGCATCCAG 720

CACGTGGCGT TCCTCACCGA CGACCTGGTC AAGACCTGGG ACGCGTTGAA GAAAATCGGC 780

ATGCGCTTCA TGACCGCGCC GCCAGACACT TATTACGAAA TGCTCGAAGG CCGCCTGCCT 840

GACCACGGCG AGCCGGTGGA TCAACTGCAG GCACGCGGTA TCCTGCTGGA CGGATCTTCC 900

GTGGAAGGCG ACAAACGCCT GCTGCTGCAG ATCTTCTCGG AAACCCTGAT GGGCCCGGTG 960

TTCTTCGAAT TCATCCAGCG CAAGGGCGAC GATGGGTTTG GCGAGGGCAA CTTCAAGGCG 1020

CTGTTCGAGT CCATCGAACG TGACCAGGTG CGTCGTGGTG TATTGACCGC CGATTAA 1077

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SEQ ID NO: 2:

AATCAATTTC ACACAGGAAA CAGCTATGAC CATGATTACG AATTCGGGCC CGGGCGCGTG 60

ATCCGGCGGC GGCAGCGGCG GCGGCGGTGC AGGCGGGTGC CGAGGAGATC GTGCTGCAGC 120

CCATCAAGGA GATCTCCGGC ACCGTCAAGC TGCCGGGGTC CAAGTCGCTT TCCAACCGGA 180

TCCTCCTACT CGCCGCCCTG TCCGAGGGGA CAACAGTGGT TGATAACCTG CTGAACAGTG 240

AGGATGTCCA CTACATGCTC GGGGCCTTGA GGACTCTTGG TCTCTCTGTC GAAGCGGACA 300

AAGCTGCCAA AAGAGCTGTA GTTGTTGGCT GTGGTGGAAA GTTCCCAGTT GAGGATGCTA 360

AAGAGGAAGT GCAGCTCTTC TTGGGGAATG CTGGAACTGC AATGCGGCCA TTGACAGCAG 420

CTGTTACTGC TGCTGGTGGA AATGCAACTT ACGTGCTTGA TGGAGTACCA AGAATGAGGG 480

AGAGACCCAT TGGCGACTTG GTTGTCGGAT TGAAGCAGCT TGGTGCAGAT GTTGATTGTT 540

TCCTTGGCAC TGACTGCCCA CCTGTTCGTG TCAATGGAAT CGGAGGGCTA CCTGGTGGCA 600

AGGTCAAGCT GTCTGGCTCC ATCAGCAGTC AGTACTTGAG TGCCTTGCTG ATGGCTGCTC 660

CTTTGGCTCT TGGGGATGTG GAGATTGAAA TCATTGATAA ATTAATCTCC ATTCCGTACG 720

TCGAAATGAC ATTGAGATTG ATGGAGCGTT TTGGTGTGAA AGCAGAGCAT TCTGATAGCT 780

GGGACAGATT CTACATTAAG GGAGGTCAAA AATACAAGTC CCCTAAAAAT GCCTATGTTG 840

AAGGTGATGC CTCAAGCGCA AGCTATTTCT TGGCTGGTGC TGCAATTACT GGAGGGACTG 900

TGACTGTGGA AGGTTGTGGC ACCACCAGTT TGCAGGGTGA TGTGAAGTTT GCTGAGGTAC 960

TGGAGATGAT GGGAGCGAAG GTTACATGGA CCGAGACTAG CGTAACTGTT ACTGGCCCAC 1020

CGCGGGAGCC ATTTGGGAGG AAACACCTCA AGGCGATTGA TGTCAACATG AACAAGATGC 1080

CTGATGTCGC CATGACTCTT GCTGTGGTTG CCCTCTTTGC CGATGGCCCG ACAGCCATCA 1140

GAGACGTGGC TTCCTGGAGA GTAAAGGAGA CCGAGAGGAT GGTTGCGATC CGGACGGAGC 1200

TAACCAAGCT GGGAGCATCT GTTGAGGAAG GGCCGGACTA CTGCATCATC ACGCCGCCGG 1260

AGAAGCTGAA CGTGACGGCG ATCGACACGT ACGACGACCA CAGGATGGCC ATGGCCTTCT 1320

CCCTTGCCGC CTGTGCCGAG GTCCCCGTCA CCATCCGGGA CCCTGGGTGC ACCCGGAAGA 1380

CCTTCCCCGA CTACTTCGAT GTGCTGAGCA CTTTCGTCAA GAATTAATAA AGCGTGCGAT 1440

ACTACCACGC AGCTTGATTG AAGTGATAGG CTTGTGCTGA GGAAATACAT TTCTTTTGTT 1500

CTGTTTTTCT CTTTCACGGG ATTAAGTTTT GAGTCTGTAA CGTTAGTTGT TTGTAGCAAG 1560

TTTCTATTTC GGATCTTAAG TTTGTGCACT GTAAGCCAAA TTTCATTTCA AGAGTGGTTC 1620

GTTGGAATAA TAAGAATAAT AAATTACGTT TCAGTGAAAA AAAAAAAAAA AAAAAAAAAA 1680

AAAAAAAAAA AAAAAAAAAA AACCCGGGAA TTC 1713

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