IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CORTEVA AGRISCIENCE LLC, Plaintiff, v. MONSANTO COMPANY and BAYER CROPSCIENCE LP, Defendants.	)	C.A. No. 22-1046-GBW **JURY TRIAL DEMANDED** [redacted] [redacted] **REDACTED VERSION**

FIRST AMENDED COMPLAINT FOR PATENT INFRINGEMENT

Plaintiff Corteva Agriscience LLC ("Corteva" or "Plaintiff"), by and through its undersigned attorneys, files this First Amended Complaint for patent infringement against Monsanto Company and Bayer CropScience LP (collectively, "Defendants"), and alleges as follows:

I. NATURE OF THE ACTION

1. This is an action for infringement of United States Patent Nos. 10,947,555 ("the '555 patent"); 11,149,283 ("the '283 patent"); and 11,299,745 ("the '745 patent") arising under the patent laws of the United States, 35 U.S.C. § 100 *et seq*.

2. The '555, '283, and '745 patents are directed to Corteva's invention of transgenic (genetically modified) plants and plant cells that produce AAD-1 enzymes conferring resistance to two different classes of herbicides with different modes of action: phenoxy auxin herbicides (e.g., 2,4-D) and (R)-aryloxyphenoxypropionate herbicides (e.g., quizalofop). Corteva's invention, commercialized in Corteva's Enlist® branded corn products, enables growers to use multiple types of herbicides to control weeds, including glyphosate-resistant weeds that are resistant to Monsanto's glyphosate herbicide, Roundup®.

Inari Exhibit 1050
Inari v. Corteva

3. With knowledge of Corteva's invention, Defendants have made and used transgenic plants that produce an AAD-1 enzyme having dual activity against 2,4-D and quizalofop. Defendants have renamed such AAD-1 enzymes as "FT" enzymes. Defendants have filed at least two applications with the U.S. Department of Agriculture seeking nonregulated status to permit marketing of transgenic plants producing an AAD-1 (FT) enzyme in the United States (i.e., MON 87429 maize and MON 94313 soybeans).

4. By making and using Corteva's patented transgenic plants in the United States, Defendants have infringed the '555, '283, and '745 patents at least under 35 U.S.C. § 271(a).

II. THE PARTIES

5. Plaintiff Corteva Agriscience LLC is incorporated in Delaware with a place of business located at Chestnut Run Plaza, 974 Centre Road, Wilmington, Delaware 19805.

6. Corteva is one of the world's largest commercial seed and plant producers. Corteva uses genetic research to develop crop plants designed to increase quantity, quality, and sustainability of yields for farmers, including herbicide-resistant transgenic maize hybrids and soybean varieties.

7. Corteva has received numerous patents in the United States and in other countries for its innovative discoveries, including the '555, '283, and '745 patents. Corteva owns, by valid assignment, all rights, title, and interest in the asserted '555, '283, and '745 patents.

8. Defendant Monsanto Company is incorporated under the laws of the State of Delaware. On information and belief, as reported in its filings with the United States Securities and Exchange Commission, Monsanto Company has a registered office at 251 Little Falls Drive, Wilmington, Delaware 19808-1674.

9. Defendant Bayer CropScience LP is organized and existing under the laws of the State of Delaware. On information and belief, Bayer CropScience LP also has a registered office at 251 Little Falls Drive, Wilmington, Delaware 19808-1674.

10. On information and belief, Defendants Monsanto Company and Bayer CropScience LP are wholly owned by Bayer AG and part of its Crop Science Division. On information and belief, Defendants are engaged in developing, producing, and selling crop seeds and plants, including herbicide-resistant transgenic plants.

III. JURISDICTION AND VENUE

11. This action arises under the patent laws of the United States, 35 U.S.C. § 100 *et seq*. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331 and 1338(a).

12. This Court has personal jurisdiction over Monsanto Company and Bayer CropScience LP, which have purposefully availed themselves of the protections of this forum by incorporating in Delaware. Monsanto Company and Bayer CropScience LP have also availed themselves of the protections of this forum by filing actions in this Court. *See, e.g.*, *Monsanto Co. v. Syngenta Seeds Inc.*, No. 1:04-cv-305 (D. Del.); *Monsanto Co. v. Mycogen Plant Sci., Inc.*, No. 1:96-cv-133 (D. Del.); *Monsanto Co. v. Pharmacia & Upjohn Co.*, No. 1:97-cv-537 (D. Del.); *Bayer CropScience LP v. United Indus. Corp.*, No. 1:03-cv-352 (D. Del.).

13. Venue is proper in this Judicial District under 28 U.S.C. §§ 1391(b) and (c) and 1400(b), at least because Monsanto Company and Bayer CropScience LP are incorporated in Delaware and because Delaware is a convenient forum for resolution of the parties' disputes set forth herein.

IV. THE PATENTS IN SUIT

A. The ’555 Patent

14. The ’555 patent, entitled “Herbicide Resistance Genes,” was duly and legally issued by the United States Patent and Trademark Office (“USPTO”) on March 16, 2021. The ’555 patent identifies Terry Wright, Justin Lira, Donald Merlo, and Nicole Arnold as inventors. A true and correct copy of the ’555 patent is attached as Exhibit A.

15. The ’555 patent issued from U.S. Appl. No. 15/288,406, which was filed on October 7, 2016, and published on January 26, 2017, as U.S. Appl. Publ. No. 2017/0022515. The ’555 patent claims priority to U.S. Appl. No. 14/820,893, filed August 7, 2015 (now U.S. Patent No. 10,174,337); U.S. Appl. No. 12/951,813, filed November 22, 2010 (now U.S. Patent No. 9,127,289); U.S. Appl. No. 11/587,893, filed May 22, 2008 (now U.S. Patent No. 7,838,733); Patent Cooperation Treaty Appl. No. PCT/US2005/014737, filed May 2, 2005; and Provisional Appl. No. 60/567,052, filed April 30, 2004.

16. The invention of the ’555 patent relates to transgenic plants having resistance to at least two different classes of herbicides: phenoxy auxin herbicides such as 2,4-D and (R)-aryloxyphenoxypropionate herbicides such as quizalofop. The ’555 patent’s transgenic plants include novel maize and soybean lines. Maize (also known as corn) and soybeans are used as human food, livestock feed, industrial raw materials, and in biofuel production. Maize and soybeans are commercially important crops in the United States.

17. As the ’555 patent states, weeds can quickly deplete soil of valuable nutrients, posing a challenge for growers of maize, soybeans, and other crops. Ex. A at 1:21-29. To control weeds, growers have commonly used the herbicide glyphosate (e.g., Roundup®). *See id.* at 1:22-29. During the 1990s, Monsanto introduced genetically engineered Roundup Ready® transgenic crops having glyphosate tolerance so that growers could use glyphosate to control weeds while

minimizing harm to their crops. *Id.* at 1:30-53. Glyphosate-tolerant transgenic crops were widely adopted, grown on over 80% of soybean acres and on over 20% of maize acres in the United States. *Id.* at 2:10-19. Over-reliance on glyphosate and glyphosate-tolerant crops, however, led to increasing prevalence of weeds having glyphosate resistance, including both broadleaf and grass weeds. *Id.* at 1:58-2:10.

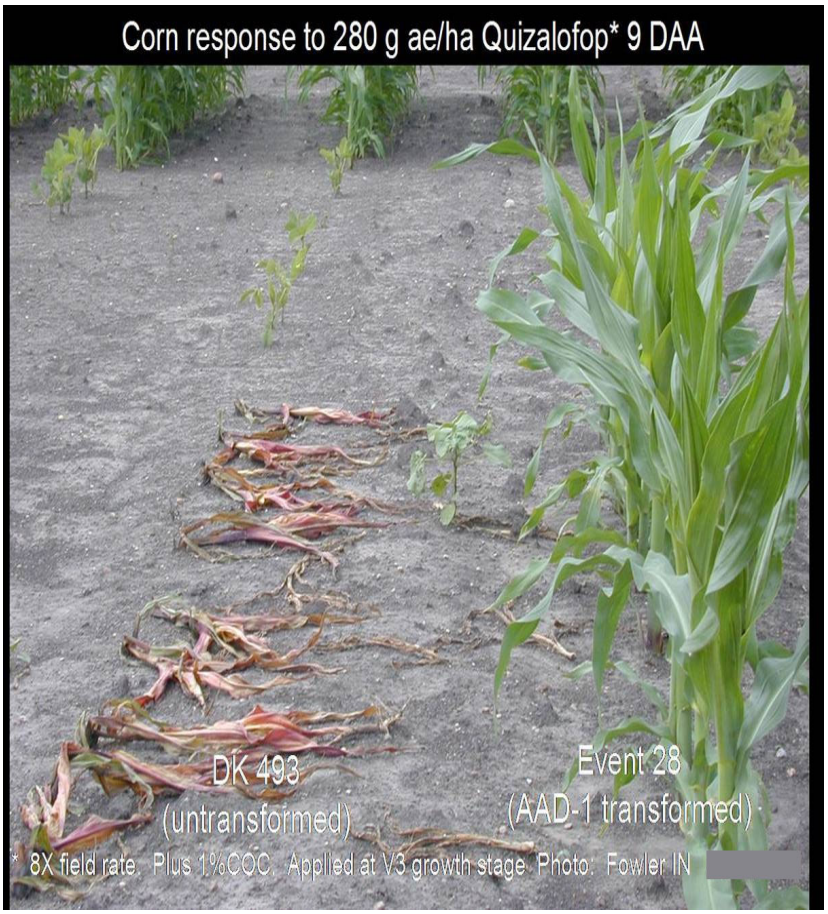
18. In areas where glyphosate-resistant weeds developed, growers could compensate for glyphosate's ineffectiveness by using other herbicides, such as 2,4-D. *Id.* at 2:20-31. But use of 2,4-D was limited due to the sensitivity of certain dicot crops, including soybeans, to this herbicide and due to injury it can cause in monocot crops, such as maize. *Id.* at 2:31-36.

19. Corteva's invention, described in the '555 patent, enables growers to use multiple types of herbicides to control weeds, including glyphosate-resistant weeds. As the '555 patent discloses, the inventors' creation of transgenic plants expressing the bacterial α-ketoglutarate-dependent ("αKG") dioxygenase enzyme RdpA from *Sphingobium herbicidovorans* resulted in a surprising discovery: these novel transgenic plants exhibited dual activity against two different classes of herbicides with different modes of action. *Id.* at 19:34-63. These two classes are (R)-aryloxyphenoxypropionate herbicides (e.g., quizalofop), which target grass weeds, and phenoxy auxin herbicides (e.g., 2,4-D), which target broadleaf weeds. *Id.* at 19:64-20:3.

20. In light of this unique dual herbicide-tolerance activity, the enzymes and genes of the '555 patent are called "AAD-1" (AryloxyAlkanoate Dioxygenase) proteins and genes. *Id.* at 19:52-54, 4:32-46. The '555 patent discloses that AAD-1 proteins beneficially provide "tolerance to combinations of herbicides that would control nearly all broadleaf and grass weeds." *Id.* at 20:13-15. The '555 patent discloses that AAD-1 genes can be "stacked" with other herbicide-

resistance genes, including glyphosate-resistance genes, glufosinate-resistance genes, and others to confer resistance to additional herbicides. *Id.* at 17:53-57, 20:15-20.

21. The ’555 patent discloses novel transgenic plants and plant cells that comprise a polynucleotide encoding an AAD-1 protein. *Id.* at 18:50-58, 38:65-39:3. These transgenic plants and plant cells include maize and soybeans. *Id.* at 20:63-21:25, 80:10-95:37 (Examples 7-9 describing transgenic maize), 102:1-106:40 (Example 13 describing transgenic soybeans). The ’555 patent reports that transgenic maize expressing an AAD-1 protein exhibits “robust field level resistance to 2,4-D and quizalofop,” thus providing growers with new solutions for controlling weeds, including glyphosate-resistant weeds. *Id.* at 92:49-50. Figure 16 of the ’555 patent, for example, depicts the field testing and quizalofop tolerance of transgenic maize expressing an AAD-1 protein as compared to control plants that do not express an AAD-1 protein:



Id. at Fig. 16.

22. The claims of the ’555 patent are generally directed to transgenic plants and plant cells comprising a polynucleotide encoding an AAD-1 protein defined by an amino acid sequence motif and 85% amino acid sequence identity to RdpA (i.e., SEQ ID NO: 9), as well as methods of controlling weeds. Claim 1, for example, recites:

> 1. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:
>
> > i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and
> >
> > ii) an AAD-1 motif having the general formula of:
> > $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein
> >
> > X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;
> > $(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;
> > $(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and
> > $(X)_{271\text{-}280}$ represents a sequence of 10 amino acids.

23. Dependent claim 6 of the ’555 patent recites: “A transgenic plant comprising a plurality of the plant cells of claim 1, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.”

B. The ’283 Patent

24. The ’283 patent, entitled “Herbicide Resistance Genes,” was duly and legally issued by the USPTO on October 19, 2021. The ’283 patent identifies Terry Wright, Justin Lira, Donald Merlo, and Nicole Arnold as inventors. A true and correct copy of the ’283 patent is attached as Exhibit G.

25. The ’283 patent is related to the ’555 patent. The ’283 patent issued from United States Appl. No. 17/175,966, which was filed on February 15, 2021 as a continuation of the

application that issued as the ’555 patent. The ’283 patent also claims priority through the same chain of applications as the ’555 patent to Patent Cooperation Treaty Appl. No. PCT/US2005/014737, filed May 2, 2005, and to Provisional Appl. No. 60/567,052, filed April 30, 2004.

26. The claims of the ’283 patent are generally directed to transgenic maize plant cells comprising a polynucleotide encoding an AAD-1 protein defined by an amino acid sequence motif and 85% amino acid sequence identity to RdpA (i.e., SEQ ID NO: 9), as well as transgenic maize plants and methods of controlling weeds. *See, e.g.*, Ex. G at claims 1, 3, and 9. Claim 1, for example, recites:

> 1. A transgenic maize plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide which are aryloxyalkanoate herbicides, further wherein said AAD-1 protein comprises:
>
> i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and
>
> ii) an AAD-1 motif having the general formula of:
>
> $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ (SEQ ID NO: 34), wherein
>
> X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;
>
> $(X)_{114-137}$ represents a sequence of 24 amino acids;
>
> $(X)_{139-269}$ represents a sequence of 131 amino acids; and
>
> $(X)_{271-280}$ represents a sequence of 10 amino acids,
>
> wherein said AAD-1 protein when expressed in a maize plant cell renders said maize plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as compared to an untransformed maize plant cell.

C. The ’745 Patent

27. The ’745 patent, entitled “Herbicide Resistance Genes,” was duly and legally issued by the USPTO on April 12, 2022. The ’745 patent identifies Terry Wright, Justin Lira, Donald Merlo, and Nicole Arnold as inventors. A true and correct copy of the ’745 patent is attached as Exhibit H.

28. The ’745 patent is related to the ’283 and ’555 patents. The ’745 patent issued from United States Appl. No. 17/403,476, which was filed on August 16, 2021 as a continuation of the application that issued as the ’283 patent. The ’745 patent also claims priority through the same chain of applications as the ’283 patent to Patent Cooperation Treaty Appl. No. PCT/US2005/014737, filed May 2, 2005, and to Provisional Appl. No. 60/567,052, filed April 30, 2004.

29. The claims of the ’745 patent are generally directed to transgenic soybean plant cells comprising a polynucleotide encoding an AAD-1 protein defined by an amino acid sequence motif and 85% amino acid sequence identity to RdpA (i.e., SEQ ID NO: 9), as well as transgenic soybean plants and methods of controlling weeds. *See, e.g.*, Ex. H at claims 1, 3, and 9. Claim 1, for example, recites:

> 1. A transgenic soybean plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, which are aryloxyalkanoate herbicides, further wherein said AAD-1 protein comprises:
>
> i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and
>
> ii) an AAD-1 motif having the general formula of:
>
> $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}$
> $H(X)_{271\text{-}280}R$ (SEQ ID NO: 34), wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids,

wherein said AAD-1 protein when expressed in a soybean plant cell renders said soybean plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as compared to an untransformed soybean plant cell.

V. DEFENDANTS' INFRINGING TRANSGENIC PLANTS ENCODING AN AAD-1 PROTEIN

30. Defendants have been aware of the '555, '283, and '745 patent family disclosing Corteva's invention of transgenic plants and plant cells comprising a recombinant polynucleotide encoding an AAD-1 protein that has dual activity against 2,4-D and quizalofop herbicides. For example, Monsanto's U.S. Patent No. 7,855,326,[1] issued December 21, 2010, cites the WO 2005/107437 publication of Corteva's Patent Cooperation Treaty Appl. No. PCT/US2005/014737, to which the '555, '283, and '745 patents claim priority. Monsanto has also cited other Corteva patents and publications within the '555, '283, and '745 patent family, including U.S. Patent No. 7,838,733 and U.S. Patent No. 9,127,289, and the publications of U.S. Appl. No. 12/951,813 (US 2011/0124503) and U.S. Appl. No. 14/820,893 (US 2015/0344903). *See, e.g.*, Monsanto U.S. Patent No. 10,023,874.

31. On January 26, 2017, Corteva's application for the '555 patent published as U.S. Appl. Publ. No. 2017/0022515 (the "2017 publication"). The claims of Corteva's 2017 publication were directed to transgenic plants and plant cells encoding a dual-activity AAD-1 protein having

[1] On information and belief, Defendant Monsanto Company is the parent of Monsanto Technology, LLC, the assignee of U.S. Patent No. 7,855,326.

the AAD-1 motif and 85% amino acid sequence identity to RdpA (i.e., SEQ ID NO: 9) and methods of controlling weeds.

32. On information and belief, Defendants used their knowledge of Corteva's invention to make transgenic plants comprising a recombinant polynucleotide encoding dual-activity AAD-1 proteins. On March 3, 2019, Defendants published certain details of their activities in an article by Clayton T. Larue et al., *Development of Enzymes for Robust Aryloxyphenoxypropionate and Synthetic Auxin Herbicide Tolerance Traits in Maize and Soybean Crops*, 75(8) PEST MGMT. SCI. 2086-94 (2019) ("Larue," attached as Exhibit B).

33. In Larue, Defendants reported that they made transgenic maize and soybean plants that express RdpA-variant proteins exhibiting dual activity against 2,4-D and quizalofop herbicides. Ex. B at 2086-87. In explaining their rationale for working with RdpA, Defendants cited an AAD-1 publication by Corteva inventor Terry Wright and his colleagues. *Id.* at 2087, 2094 (citing ref. 9: T.R. Wright et al., *Robust Crop Resistance to Broadleaf and Grass Herbicides Provided by Aryloxyalkanoate Dioxygenase Transgenes*, 107 PROC. NAT'L ACAD. SCI. USA 20240-45 (2010)). Rather than using Corteva's AAD-1 name, Defendants renamed the RdpA homologues as "FT" enzymes (e.g., FT_T and FT_Tv7 enzymes). *Id.* at 2087, 2090, 2092-94.

34. In Larue, Defendants described their field trials using transgenic maize plants expressing an FT_T protein. Defendants reported that the transgenic maize plants exhibited dual herbicide resistance against quizalofop and 2,4-D as compared to control maize plants that did not express an FT_T protein. *Id.* at 2092-94. Those field tests were performed in Illinois. *Id.* at 2088.



Control FT_T

Treatment: 16x QFOP (1440 g/ha) at both V2 and V4 growth stages.



Control FT_T

Treatment: 16x 2,4-D (18000 g/ha) at both V2 and V4 growth stages.

Id. at 2090 (Fig. 3(b) and 3(d)), 2093 ("In the treatments with QFOP [quizalofop], the control plants were completely killed while . . . [plants expressing FT_T] showed little injury . . . . In treatments with 2,4-D, a similar trend as with QFOP was observed . . . .").

35. Defendants also reported in Larue that they transformed a "commercially relevant soybean line" to express FT_T and other FT proteins (FT_Tv7 and FT_Tv3) having dual activity against a synthetic auxin herbicide such as 2,4-D and an aryloxyphenoxypropionate ("FOP") herbicide such as quizalofop. *Id.* at 2094. Defendants reported that these FT proteins exhibited "FOP activity . . . suitable for robust in-plant tolerance" and "demonstrated 2,4-D tolerance in soybean [plants]." *Id.* at 2092, 2094, 2092 (Table 1), 2093 (Fig. 6). Defendants also reported that, in particular, transgenic soybean plants expressing the FT_Tv7 protein showed "commercially relevant" 2,4-D tolerance in field applications. *Id*. at 2094.

36. Defendants reported in Larue that the amino acid sequences of the FT_T and FT_Tv7 proteins were published in GenBank with the accession numbers MH043112 and MH043115, respectively. *Id.* at 2088.

37. On information and belief, the FT_T protein's amino acid sequence has at least 85% sequence identity with SEQ ID NO: 9 recited in the '555 and '283 patent claims. On information and belief, the FT_T protein also has the AAD-1 motif as annotated below.

GenBank Accession No. MH043112 (FT_T Sequence)

MHAALTPLTNKYRFIDVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRF
GPVDPVPILKSIEGYPEVQMIRREANESSRFIGDDWHTDSTFLDAPPAAVVMRAIEVPEYGGDTGFLSMYSAWE
TLSPTMQATIEGLNVVHSATKVFGSLYQATNWRFSNTSVKVMDVDAGDRETVHPLVVTHPVTGRRALYCNQVYC
QKIQGMTDAESKSLLQFLYEHATKFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVAGDKPSR

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$

FIGURE 1

Ex. C (annotations added).

38. On information and belief, the FT_Tv7 protein's amino acid sequence has at least 85% sequence identity with SEQ ID NO: 9 recited in the '555 and '745 patent claims. On information and belief, the FT_Tv7 protein also has the AAD-1 motif as annotated below.

GenBank Accession No. MH043115 (FT_Tv7 Sequence)

MHAALTPLTNKYRFIDVQPLTGVLGAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRF
GPVDPVPILKSIEGYPEVQMIRREANESSRYIGDDWHADSTFLDAPPAAVVMRAIEVPEYGGDTGFLSMYSAWE
TLSPTMQATIEGLNVVHSATKVFGSLYQATNWRFSNTSVKVMDVDAGDRETVHPLVVTHPVTGRRALYCNQVYC
QKIQGMTDAESKSLLQFLYEHATQFDFTCRVRWKKDQVLVWDNLCTMHRAVPDYAGKFRYLTRTTVAGDKPSR

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$

FIGURE 2

Ex. D (annotations added).

39. Shortly after publishing Larue, Defendants submitted a Petition to the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture ("APHIS Petition," attached as Exhibit E) on June 27, 2019. On information and belief, Defendants' APHIS Petition requests a determination of nonregulated status to permit marketing of maize containing the MON 87429

transgenic event, which encodes the dual-activity FT_T protein Defendants previously described in Larue. Ex. E at 25, 85 ("The FT_T protein produced in MON 87429 is encoded by the *ft_t* gene . . . .").[2]

40. In the APHIS Petition, Defendants again report that the FT_T protein exhibits the AAD-1 dual activity of degrading a phenoxy auxin herbicide (e.g., 2,4-D) and an (R)-aryloxyphenoxypropionate herbicide (e.g., quizalofop). For example, Defendants state that the FT_T protein degrades quizalofop and 2,4-D into "herbicidally-inactive" compounds. *Id.* at 86; *see also id.* at 43 ("[The] FT_T protein confers tolerance to quizalofop and 2,4-D herbicides.").

41. On information and belief, Defendants' APHIS Petition illustrates that the FT_T protein comprises an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9 (i.e., the sequence of RdpA) recited in the '555 and '283 patent claims. Defendants reported in the APHIS Petition that the "amino acid sequence of the FT_T protein shares ~ 89% sequence identity with wild type RdpA." *Id.* at 85.

42. Defendants' APHIS Petition also illustrates that the FT_T protein comprises the structural AAD-1 motif $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$ recited in the '555 and '283 patent claims. In the APHIS Petition, Defendants disclose the amino acid sequence of the FT_T protein, which on information and belief contains the AAD-1 motif as annotated below.

[2] The *ft_t* gene may be stacked with other genes encoding proteins conferring resistance to five or more different herbicides. Ex. E at 25 (APHIS Petition: "The flexibility to use dicamba, glufosinate, quizalofop, 2,4-D and glyphosate and/or combinations of these five herbicides . . . will provide an effective weed management system for maize production.").

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1    AMHAALTPLT NKYRFIDVQP LTGVLGAEIT GVDLREPLDD STWNEILDAF
51   HTYQVIYFPG QAITNEQHIA FSRRFGPVDP VPILKSIEGY PEVQMIRREA
101  NESSRFIGDD WHTDSTFLDA PPAAVVMRAI EVPEYGGDTG FLSMYSAWET
151  LSPTMQATIE GLNVVHSATK VFGSLYQATN WRFSNTSVKV MDVDAGDRET
201  VHPLVVTHPV TGRRALYCNQ VYCQKIQGMT DAESKSLLQF LYEHATKFDF
251  TCRVRWKKDQ VLVWDNLCTM HRAVPDYAGK FRYLTRTTVA GDKPSR
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Figure V-2 Deduced Amino Acid Sequence of the FT_T Protein

$$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$$

FIGURE 3

See Ex. E at 86 (Fig. V-2) (color annotations added).

43. Defendants have also filed regulatory applications relating to MON 94313 soybeans, which, on information and belief, correspond to Defendants' HT4 soybeans. Ex. I; Ex. J; Ex. K. Defendants' regulatory filings illustrate that MON 94313 (HT4) soybeans express the FT_Tv7 protein previously described in the Larue publication, renamed as FT_T.1. Ex. K at BAYER-0000339; Ex. J at COR-AAD1-00007190 ("FT_T was further modified to enhance its enzymatic activity to 2,4-D herbicide. The result was FT_T.1, which provides the 2,4-D tolerance in MON 94313 soybean necessary for in-crop herbicide applications (Larue *et al*., 2019); FT_T.1 in this publication is referred to as FT_Tv7.").

44. On information and belief, Defendants' regulatory filings illustrate that the FT_T.1 protein comprises an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9 (i.e., the sequence of RdpA) recited in the '555 and '745 patent claims. Defendants reported that the [redacted] Ex. K at BAYER-0000338.

45. Defendants' regulatory filings also illustrate that the FT_T.1 protein comprises the structural AAD-1 motif $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$ recited in the '555 and '745 patent claims. In their regulatory filings, Defendants disclosed the amino acid sequence of the FT_T.1 protein, which on information and belief contains the AAD-1 motif as annotated below.

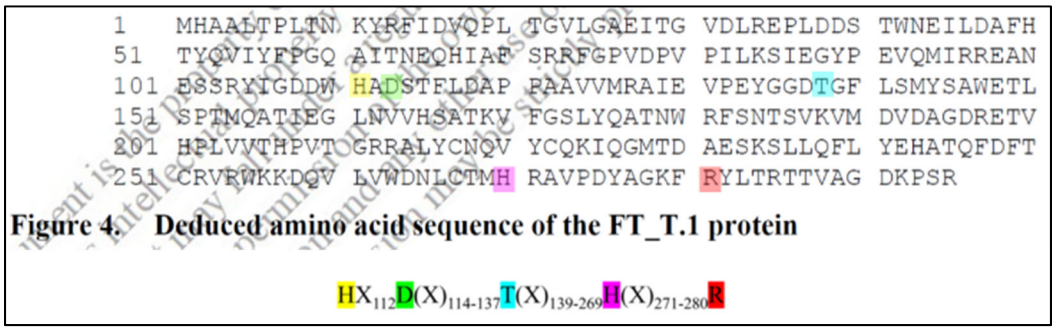


Figure 4. Deduced amino acid sequence of the FT_T.1 protein

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$

Figure 4

Ex. J at COR-AAD1-00007181 (annotations added); *see supra* ¶ 38, Fig. 2.

46. On information and belief, Defendants have continued making and using transgenic plants and plant cells having genes that encode dual-activity FT proteins in the United States since the '555, '283, and '745 patents issued on March 16, 2021, October 19, 2021, and April 12, 2022, respectively. Defendants' infringing plants and plant cells include at least HT4 (MON 87429) maize and HT4 (MON 94313) soybeans.

47. Maize: On information and belief, Defendants have continued making and using transgenic maize plants and plant cells having genes that encode dual-activity FT proteins in the United States. On April 28, 2021, for example, an article reported updates on Defendants' APHIS Petition for MON 87429 maize (also called "HT4" maize) and Defendants' continuing development efforts. Emily Unglesbee, *Five-Herbicide Corn Tech: Bayer's Future Five-Way Herbicide-Tolerant Corn Under Review by USDA*, PROGRESSIVE FARMER DTN (Apr. 28, 2021), *available at* https://www.dtnpf.com/agriculture/web/ag/crops/article/2021/04/28/bayers-future-five-way-herbicide (attached as Exhibit F). It states that "Bayer is developing a corn technology

that would tolerate in-season applications of five herbicides -- dicamba, 2,4-D, glufosinate, glyphosate and quizalofop." *Id.* It further states that "Bayer has been working on this new, five-way traited corn, which it calls HT4, and is re-focusing much of its research efforts there." *Id.* The article quoted Defendants as stating that "we . . . are focusing our resources on our fourth generation herbicide-tolerant corn (HT4) product." *Id.* The article also quoted Defendants as having paused work on an earlier generation maize product (HT3) in favor of focusing their resources on HT4 maize. *Id.* On information and belief, Defendants' APHIS Petition currently remains pending at the U.S. Department of Agriculture and has not been withdrawn.

48. [redacted]

[redacted]

49. Soybeans: On information and belief, Defendants have also continued making and using transgenic soybean plants and plant cells having genes that encode dual-activity FT proteins in the United States, including HT4 (MON 94313) soybeans. [redacted] Ex. M at BAYER-0341605; Ex. J at COR-AAD1-00007190; Ex. K at BAYER-0000338; Ex. B at 2094. Further, in their regulatory filings, Defendants also reported that "MON 94313 has been field tested in the US since 2020" and that from 2020-2022 Bayer had released MON 94313 to "major soybean growing regions" of the United States for "[r]egulatory, efficacy, yield testings, breeding, product development, demonstration trials, and nurseries." Ex. I at COR-AAD1-00007230-31.

COUNT I: INFRINGEMENT OF THE '555 PATENT

50. Corteva repeats and realleges each of the foregoing paragraphs as if fully set forth herein.

51. On information and belief, after issuance of the '555 patent, Defendants have infringed at least claims 1 and 6 of the '555 patent under 35 U.S.C. § 271(a), either literally or under the doctrine of equivalents, by making and using claimed transgenic plants and plant cells comprising a recombinant polynucleotide encoding a dual-activity AAD-1 protein without authority in the United States.

52. On information and belief, Defendants have made and used transgenic plant cells comprising a recombinant polynucleotide that encodes an AAD-1 protein, as required by claim 1

of the '555 patent, including transgenic maize and soybean cells. For example, on information and belief, Defendants' transgenic maize plants contain transgenic maize cells comprising a recombinant *ft_t* gene that encodes an AAD-1 protein, renamed by Defendants as "FT_T." Ex. E at 85 ("The FT_T protein produced in MON 87429 is encoded by the *ft_t* gene . . . ."); Ex. B at 2087-88, 2093; Ex. F; Ex. L at BAYER-0198493, 496-500. On information and belief, Defendants' HT4 (MON 94313) soybean plants contain transgenic soybean cells comprising a recombinant *ft_t.1* gene that encodes an AAD-1 protein, renamed by Defendants as "FT_T.1" (also known as FT_Tv7). Ex. K at BAYER-0000338 [redacted]); Ex. J at COR-AAD1-00007190 ("FT_T.1 in [the Larue] publication is referred to as FT_Tv7."); Ex. B at 2087-88, 2093.

53. On information and belief, the encoded FT_T and FT_T.1 (FT_Tv7) proteins each exhibit aryloxyalkanoate dioxygenase activity in which the activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as required by claim 1 of the '555 patent. On information and belief, the FT_T and FT_T.1 (FT_Tv7) proteins have the AAD-1 dual activity of enzymatically degrading 2,4-D (a phenoxy auxin herbicide) and quizalofop (an (R)-aryloxyphenoxypropionate herbicide) into "herbicidally-inactive" compounds. Ex. E at 85, 86, 43 ("[The] FT_T protein confers tolerance to quizalofop and 2,4-D herbicides."); Ex. B at 2093; Ex. L at BAYER-0198493, 496-500; Ex. K at BAYER-0000339 [redacted]); Ex. B at 2092-94 (FT_Tv7's "FOP activity was suitable for robust in-plant tolerance" and "demonstrated 2,4-D tolerance in soybean [plants]")

54. On information and belief, the encoded FT_T and FT_T.1 (FT_Tv7) proteins each comprise an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9, as

required by claim 1 of the '555 patent. Ex. E at 85 ("The amino acid sequence of the FT_T protein shares ~ 89% sequence identity with wild type RdpA . . . ."); Ex. J at COR-AAD1-00007181; Ex. K at BAYER-0000338 [redacted]).

55. On information and belief, the encoded FT_T and FT_T.1 (FT_Tv7) proteins also each comprise an AAD-1 motif having the general formula $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$ relative to the sequence of SEQ ID NO: 9, as required by claim 1 of the '555 patent. *See supra* Figs. 1-4.

56. On information and belief, Defendants have made and used a transgenic plant comprising a plurality of the plant cells of claim 1, in which expression of the recombinant polynucleotide renders the plant tolerant to an aryloxyalkanoate herbicide, as required by claim 6 of the '555 patent, including transgenic maize and soybean plants. On information and belief, expression of the *ft_t* gene of Defendants' transgenic maize plants produces the FT_T protein, rendering the transgenic plants tolerant to an aryloxyalkanoate herbicide, such as quizalofop or 2,4-D. Ex. E at 4 ("[T]he *ft_t* gene . . . expresses a FOPs and 2,4-D dioxygenase protein (FT_T) that confers tolerance to quizalofop and 2,4-D herbicides."); *id.* at 85 ("The FT_T protein produced in MON 87429 is encoded by the *ft_t* gene that provides tolerance to aryloxyalkanoate herbicides."), 43, 86; Ex. B at 2093-94; Ex. L at BAYER-0198493, 496-500. On information and belief, expression of the *ft_t.1* gene of Defendants' transgenic soybean plants produces the FT_T.1 (FT_Tv7) protein, rendering the transgenic plants tolerant to an aryloxyalkanoate herbicide, such as FOP herbicides or 2,4-D. Ex. K at BAYER-0000339 [redacted] Ex. B at 2092-

94 (FT_Tv7's "FOP activity was suitable for robust in-plant tolerance" and "demonstrated 2,4-D tolerance in soybean [plants]"); Ex. I at COR-AAD1-00007230-31; Ex. M at BAYER-0341605.

COUNT II: INFRINGEMENT OF THE '283 PATENT

57. Corteva repeats and realleges each of the foregoing paragraphs as if fully set forth herein.

58. On information and belief, after issuance of the '283 patent, Defendants have infringed at least claims 1 and 3 of the '283 patent under 35 U.S.C. § 271(a), either literally or under the doctrine of equivalents, by making and using claimed transgenic maize plants and plant cells comprising a recombinant polynucleotide encoding a dual-activity AAD-1 protein without authority in the United States. Ex. L at BAYER-0198493, 496-500.

59. On information and belief, Defendants have made and used transgenic maize plant cells comprising a recombinant polynucleotide that encodes an AAD-1 protein, as required by claim 1 of the '283 patent. For example, on information and belief, Defendants' transgenic maize plants contain transgenic maize cells comprising a recombinant *ft_t* gene that encodes an AAD-1 protein, renamed by Defendants as an "FT" protein, specifically "FT_T." Ex. E at 85 ("The FT_T protein produced in MON 87429 is encoded by the *ft_t* gene . . . ."); Ex. B at 2087-88, 2093; Ex. F; Ex. L at BAYER-0198493, 496-500.

60. On information and belief, the encoded FT_T protein exhibits aryloxyalkanoate dioxygenase activity in which the activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as required by claim 1 of the '283 patent. On information and belief, the FT_T protein has the AAD-1 dual activity of enzymatically degrading 2,4-D (a phenoxy auxin herbicide) and quizalofop (an (R)-aryloxyphenoxypropionate herbicide)

into "herbicidally-inactive" compounds. Ex. E at 85, 86, 43 ("[The] FT_T protein confers tolerance to quizalofop and 2,4-D herbicides."); Ex. B at 2093; Ex. L at BAYER-0198493, 496-500.

61. On information and belief, the encoded FT_T protein comprises an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9, as required by claim 1 of the '283 patent. Ex. E at 85 ("The amino acid sequence of the FT_T protein shares ~ 89% sequence identity with wild type RdpA . . . .").

62. On information and belief, the encoded FT_T protein also comprises an AAD-1 motif having the general formula $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$ relative to the sequence of SEQ ID NO: 9, as required by claim 1 of the '283 patent. *See supra* Figs. 1, 3.

63. On information and belief, the FT_T protein, when expressed in a maize plant cell, renders the maize plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as compared to an untransformed maize plant cell, as required by claim 1 of the '283 patent. On information and belief, compared to untransformed maize plant cells, expression of the FT_T protein renders the HT4 (MON 87429) plants tolerant to herbicides including quizalofop and 2,4-D. Ex. E at 4 ("[T]he *ft_t* gene . . . expresses a FOPs and 2,4-D dioxygenase protein (FT_T) that confers tolerance to quizalofop and 2,4-D herbicides."); *id.* at 85 ("The FT_T protein produced in MON 87429 is encoded by the *ft_t* gene that provides tolerance to aryloxyalkanoate herbicides."), 43, 86; Ex. B at 2093-94; Ex. L at BAYER-0198493, 496-500.

64. On information and belief, Defendants have made and used a transgenic maize plant comprising a plurality of the plant cells of claim 1, in which expression of the recombinant polynucleotide renders the maize plant tolerant to an aryloxyalkanoate herbicide, as required by claim 3 of the '283 patent. On information and belief, expression of the *ft_t* gene of Defendants'

transgenic maize plants produces the FT_T protein, rendering the transgenic maize plants tolerant to an aryloxyalkanoate herbicide, such as quizalofop or 2,4-D. Ex. E at 4 ("[T]he *ft_t* gene . . . expresses a FOPs and 2,4-D dioxygenase protein (FT_T) that confers tolerance to quizalofop and 2,4-D herbicides."); *id.* at 85 ("The FT_T protein produced in MON 87429 is encoded by the *ft_t* gene that provides tolerance to aryloxyalkanoate herbicides."), 43, 86; Ex. B at 2093-94; Ex. L at BAYER-0198493, 496-500.

COUNT III: INFRINGEMENT OF THE '745 PATENT

65. Corteva repeats and realleges each of the foregoing paragraphs as if fully set forth herein.

66. On information and belief, after issuance of the '745 patent, Defendants have infringed at least claims 1 and 3 of the '745 patent under 35 U.S.C. § 271(a), either literally or under the doctrine of equivalents, by making and using claimed transgenic soybean plants and plant cells comprising a recombinant polynucleotide encoding a dual-activity AAD-1 protein without authority in the United States. Ex. M at BAYER-0341605; Ex. I at COR-AAD1-00007230-31.

67. On information and belief, Defendants have made and used transgenic soybean plant cells comprising a recombinant polynucleotide that encodes an AAD-1 protein, as required by claim 1 of the '745 patent. For example, on information and belief, Defendants' transgenic soybean plants contain transgenic soybean cells comprising a recombinant *ft_t.1* gene that encodes an AAD-1 protein, renamed by Defendants as an "FT" protein, specifically "FT_T.1" (also known as FT_Tv7). Ex. K at BAYER-0000338 [redacted]); Ex. J at COR-AAD1-00007190 ("FT_T.1 in [the Larue] publication is referred to as FT_Tv7."); Ex. B at 2087-88, 2093.

68. On information and belief, the encoded FT_T.1 protein exhibits aryloxyalkanoate dioxygenase activity in which the activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as required by claim 1 of the '745 patent. On information and belief, the FT_T.1 protein has the AAD-1 dual activity of enzymatically degrading 2,4-D (a phenoxy auxin herbicide) and FOP herbicides (e.g., (R)-aryloxyphenoxypropionate herbicides) into "herbicidally-inactive" compounds. Ex. K at BAYER-0000339 [redacted]); Ex. B at 2092-94 (FT_Tv7's "FOP activity was suitable for robust in-plant tolerance" and "demonstrated 2,4-D tolerance in soybean [plants]").

69. On information and belief, the encoded FT_T.1 (FT_Tv7) protein comprises an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9, as required by claim 1 of the '745 patent. *Supra* Figs. 2, 4; Ex. J at COR-AAD1-00007181; Ex. K at BAYER-0000338 [redacted]).

70. On information and belief, the encoded FT_T.1 (FT_Tv7) protein also comprises an AAD-1 motif having the general formula $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$ relative to the sequence of SEQ ID NO: 9, as required by claim 1 of the '745 patent. *See supra* Figs. 2, 4.

71. On information and belief, the FT_T.1 protein, when expressed in a soybean plant cell, renders the soybean plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as compared to an untransformed soybean plant cell, as required by claim 1 of the '745 patent. On information and belief, compared to untransformed soybean plant cells, expression of the FT_T.1 protein renders Defendants' transgenic soybean plant cells tolerant to herbicides including FOP herbicides and 2,4-D. Ex. K at BAYER-0000339

[redacted]); Ex. B at 2092-94 (FT_Tv7's "FOP activity was suitable for robust in-plant tolerance" and "demonstrated 2,4-D tolerance in soybean [plants]").

72. On information and belief, Defendants have made and used a transgenic soybean plant comprising a plurality of the plant cells of claim 1, in which expression of the recombinant polynucleotide renders the soybean plant tolerant to an aryloxyalkanoate herbicide, as required by claim 3 of the '745 patent. On information and belief, expression of the *ft_t.1* gene of Defendants' transgenic soybean plants produces the FT_T.1 (FT_Tv7) protein, rendering the transgenic soybean plants tolerant to an aryloxyalkanoate herbicide, such as FOP herbicides or 2,4-D. Ex. K at BAYER-0000339 [redacted]); Ex. B at 2092-94 (FT_Tv7's "FOP activity was suitable for robust in-plant tolerance" and "demonstrated 2,4-D tolerance in soybean [plants]"); Ex. I at COR-AAD1-00007230-31; Ex. M at BAYER-0341605.

PRAYER FOR RELIEF

WHEREFORE, Plaintiff requests that the Court enter judgment in its favor and against Defendants as follows:

A. A judgment that Defendants have infringed at least one claim of each of the '555, '283, and '745 patents under at least 35 U.S.C. § 271(a);

B. An award of damages adequate to compensate Plaintiff for Defendants' past infringement and any continuing or future infringement, including at minimum reasonable

royalties, together with interest, costs, expenses, and disbursements as justified under 35 U.S.C. § 284;

C. An order enjoining Defendants, their officers, agents, servants, employees, attorneys, all parent and subsidiary corporations and affiliates, their assigns and successors in interest, and those persons in active concert or participation or privity with Defendants who receive notice of the injunction, from acts of infringement of the '555, '283, and '745 patents;

D. An order finding that this is an exceptional case and awarding to Plaintiff its reasonable attorneys' fees and costs pursuant to 35 U.S.C. § 285; and

E. Such other and further relief in law or equity as the Court deems just and appropriate.

DEMAND FOR JURY TRIAL

Pursuant to Federal Rule of Civil Procedure 38(b), Plaintiff demands a trial by jury on all claims and issues so triable.

Respectfully submitted,

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Dated: April 17, 2023
Redacted Version filed on April 28, 2023

EXHIBIT A



B255866

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

June 2, 2022

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

PATENT NUMBER: *10,947,555*
ISSUE DATE: *March 16, 2021*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

W. Montgomery

Wanda Montgomery
Certifying Officer

US010947555B2

(12) **United States Patent**
Wright et al.

(10) **Patent No.: US 10,947,555 B2**
(45) **Date of Patent: *Mar. 16, 2021**

(54) **HERBICIDE RESISTANCE GENES**

(71) Applicant: **Dow AgroSciences LLC**, Indianapolis, IN (US)

(72) Inventors: **Terry Wright**, Westfield, IN (US); **Justin Lira**, Zionsville, IN (US); **Donald Merlo**, Carmel, IN (US); **Nicole Arnold**, Indianapolis, IN (US)

(73) Assignee: **Dow AgroSciences LLC**, Indianapolis, IN (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 257 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **15/288,406**

(22) Filed: **Oct. 7, 2016**

(65) **Prior Publication Data**

US 2017/0022515 A1 Jan. 26, 2017

Related U.S. Application Data

(63) Continuation of application No. 14/820,893, filed on Aug. 7, 2015, now Pat. No. 10,174,337, which is a continuation of application No. 12/951,813, filed on Nov. 22, 2010, now Pat. No. 9,127,289, which is a continuation of application No. 11/587,893, filed as application No. PCT/US2005/014737 on May 2, 2005, now Pat. No. 7,838,733.

(60) Provisional application No. 60/567,052, filed on Apr. 30, 2004.

(51) **Int. Cl.**
C12N 15/82 (2006.01)
C12N 9/02 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 15/8274*** (2013.01); ***C12N 9/0069*** (2013.01); ***C12N 9/0071*** (2013.01); ***C12N 15/8209*** (2013.01)

(58) **Field of Classification Search**
CPC C12N 9/0069
See application file for complete search history.

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Primary Examiner — David H Kruse
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(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.

34 Claims, 24 Drawing Sheets

Specification includes a Sequence Listing.

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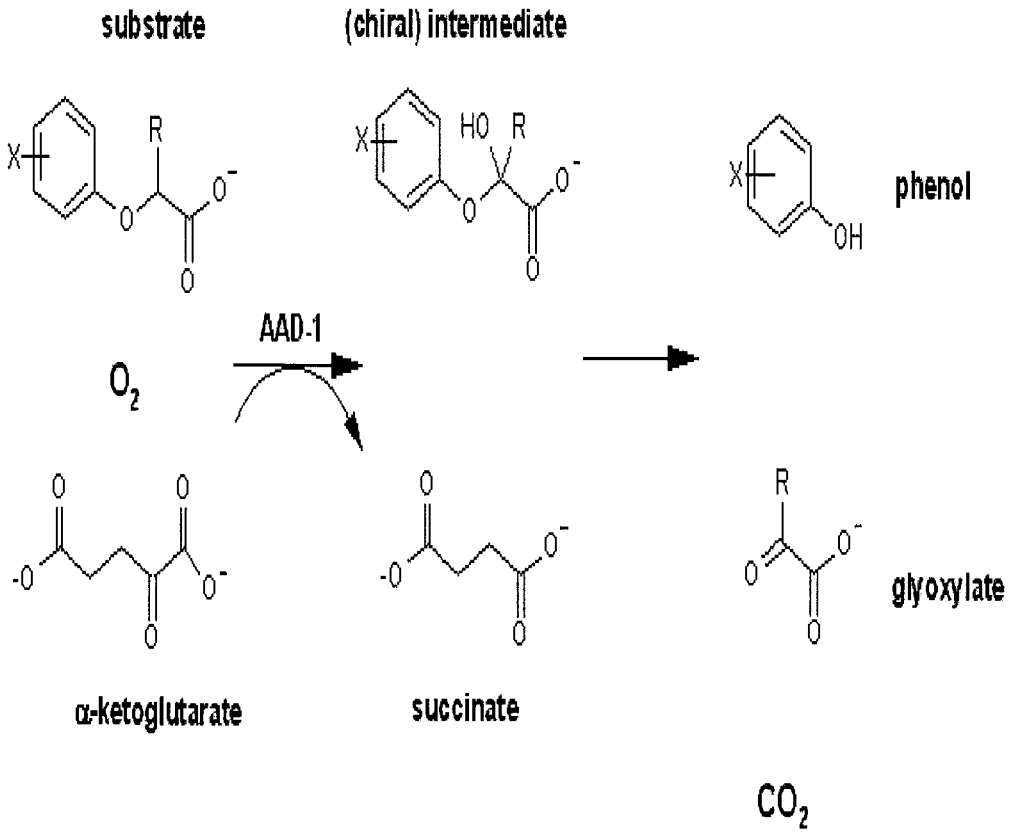
Final Decision in Opposition filed against EP 10012200.1, filed on Aug. 1, 2016.

Final Decision in Opposition filed against EP 10012201.9, filed on Aug. 1, 2016.

Final Decision in Opposition filed against EP 10012202.7, filed on Aug. 1, 2016.

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* cited by examiner



- Addition of O_2 is *stereospecific*
- Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

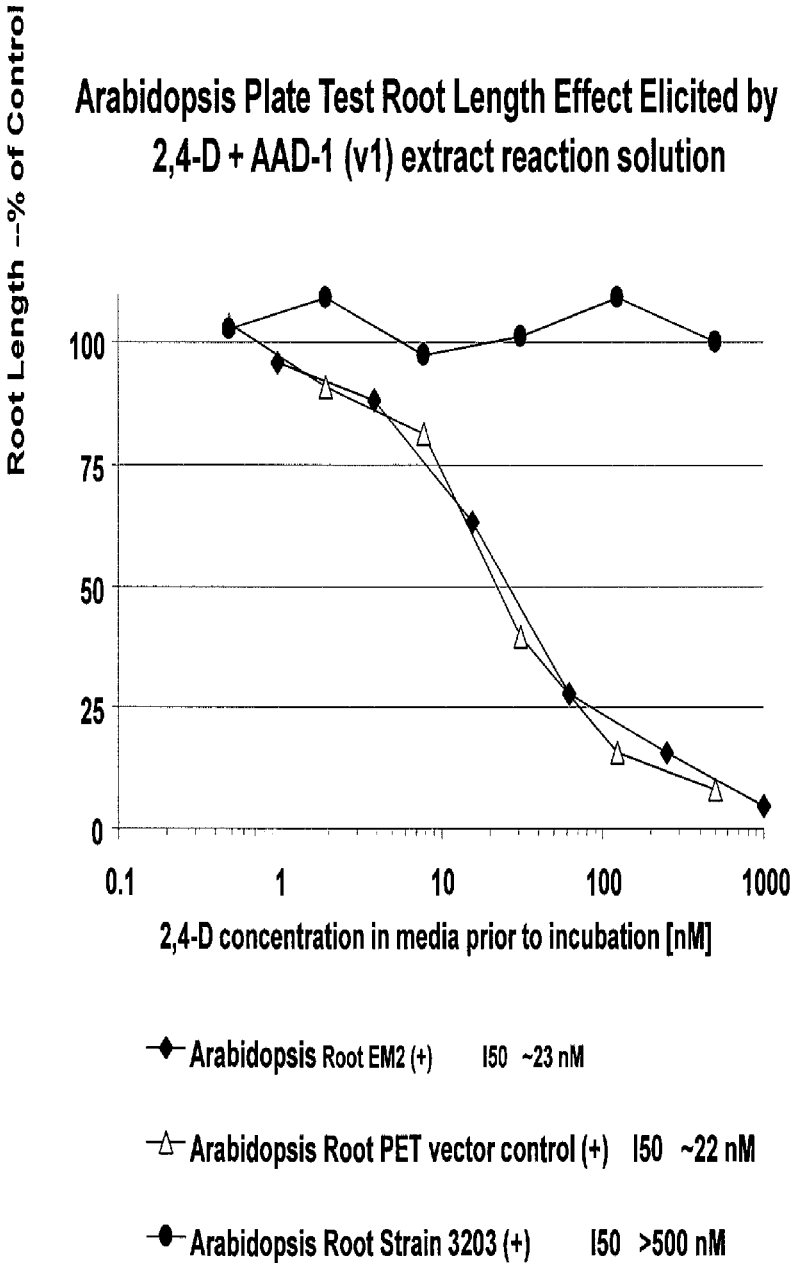

Arabidopsis Plate Test Root Length Effect Elicited by
2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

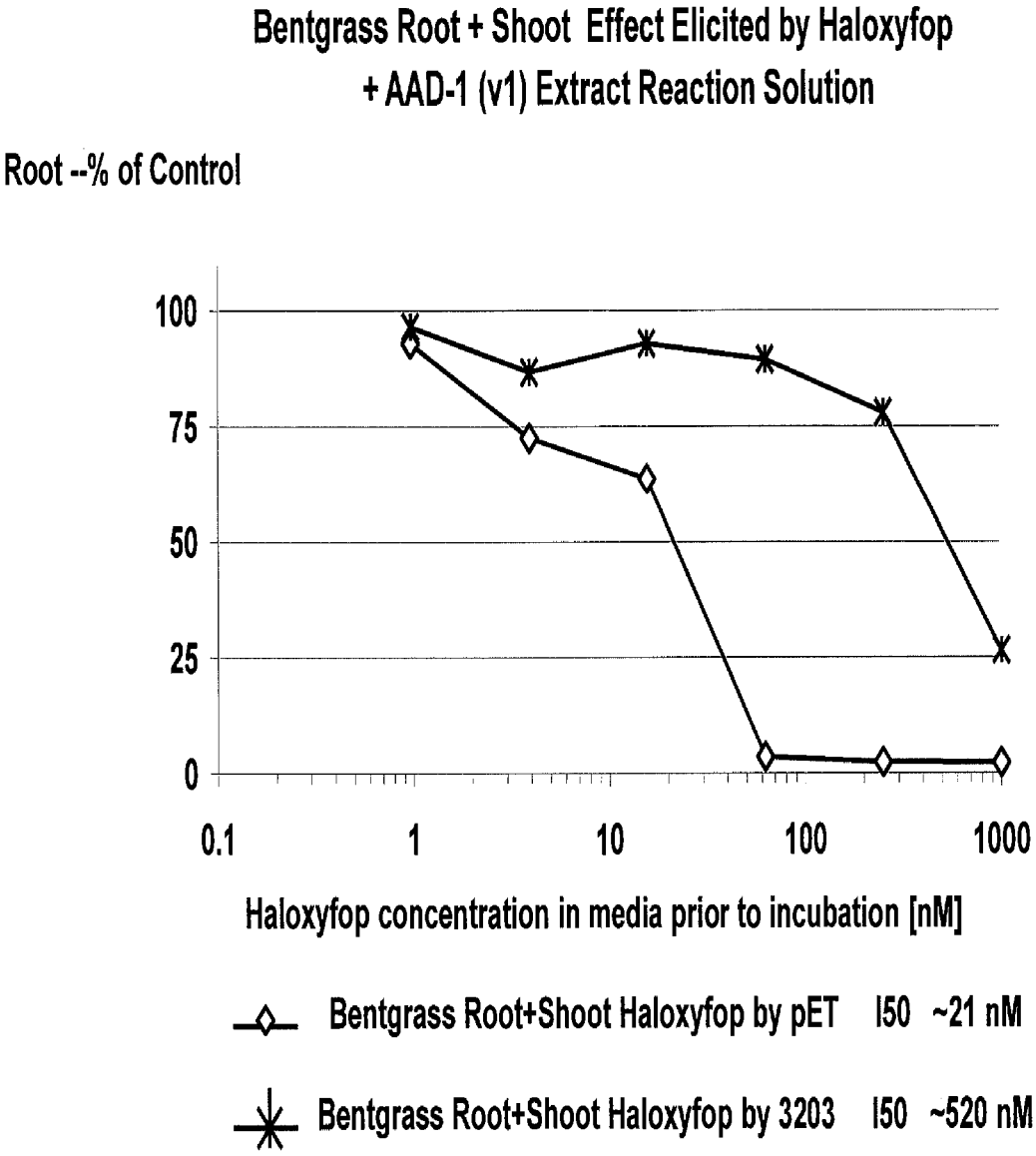

Bentgrass Root + Shoot Effect Elicited by Haloxyfop + AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

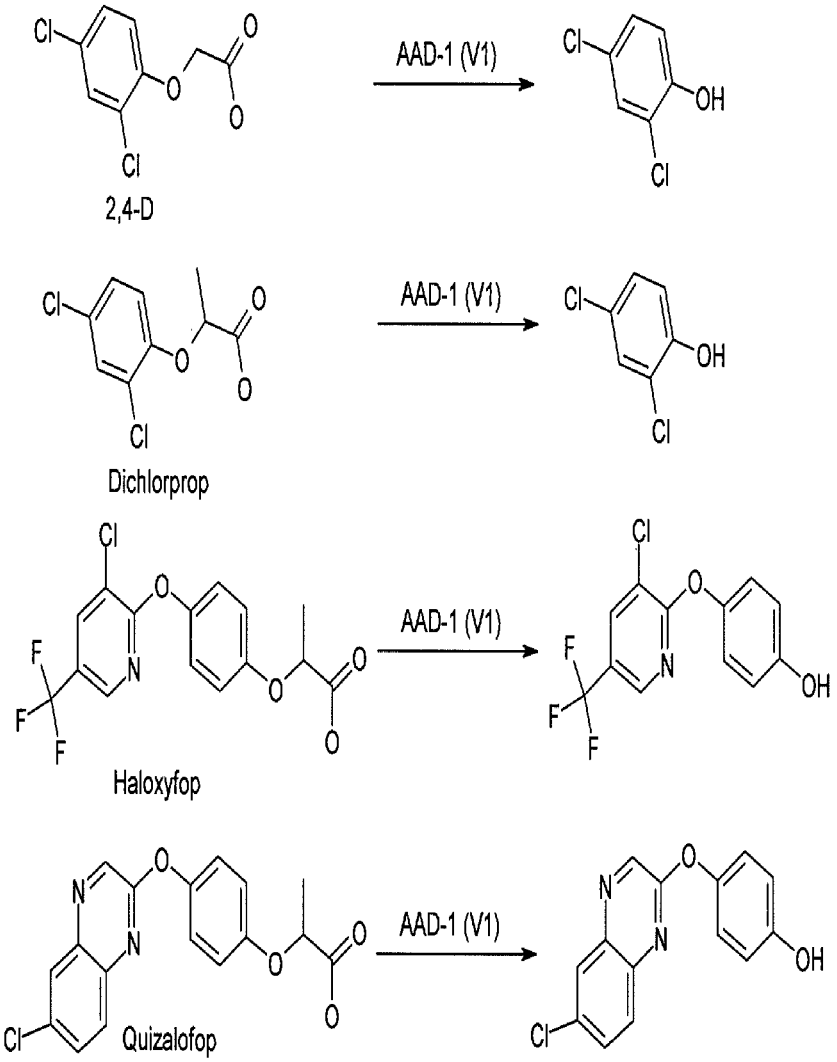

AAD-1 (V1)
2,4-D
AAD-1 (V1)
Dichlorprop
AAD-1 (V1)
Haloxyfop
AAD-1 (V1)
Quizalofop

Fig. 4

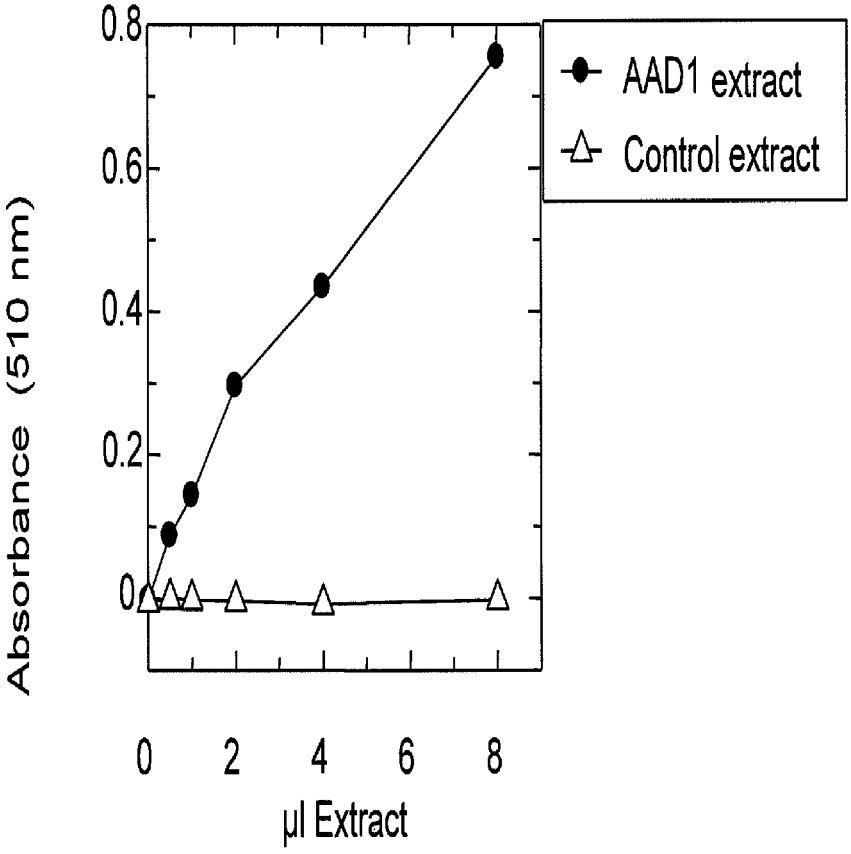

0.8
0.6
0.4
0.2
0
Absorbance (510 nm)
0
2
4
6
8
µl Extract
AAD1 extract
Control extract

Fig. 5

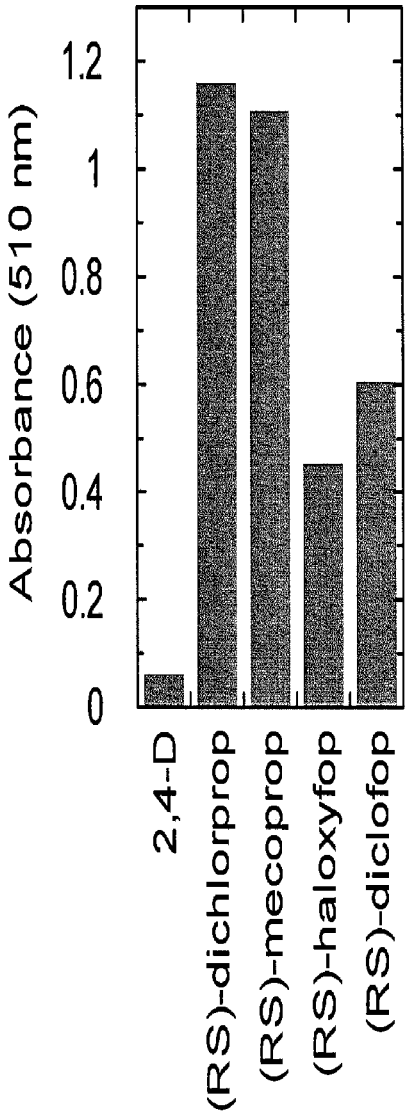

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
2,4-D
(RS)-dichlorprop
(RS)-mecoprop
(RS)-haloxyfop
(RS)-diclofop

Fig. 6A

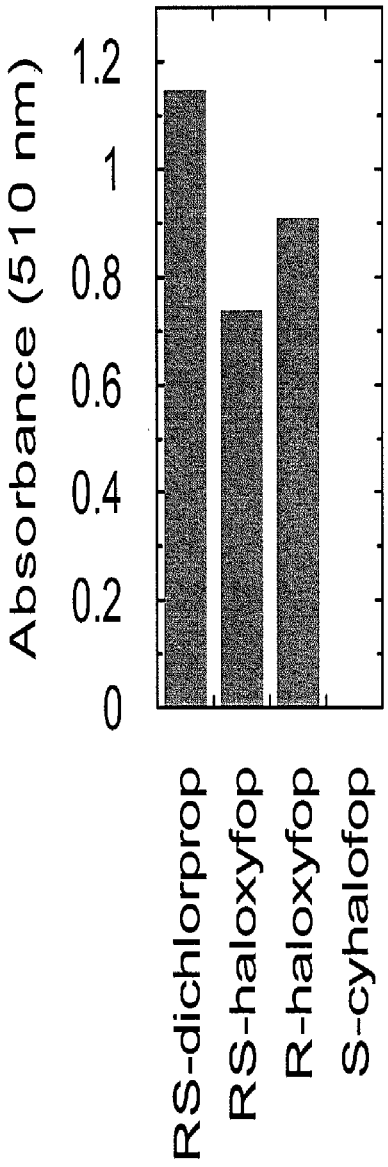

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

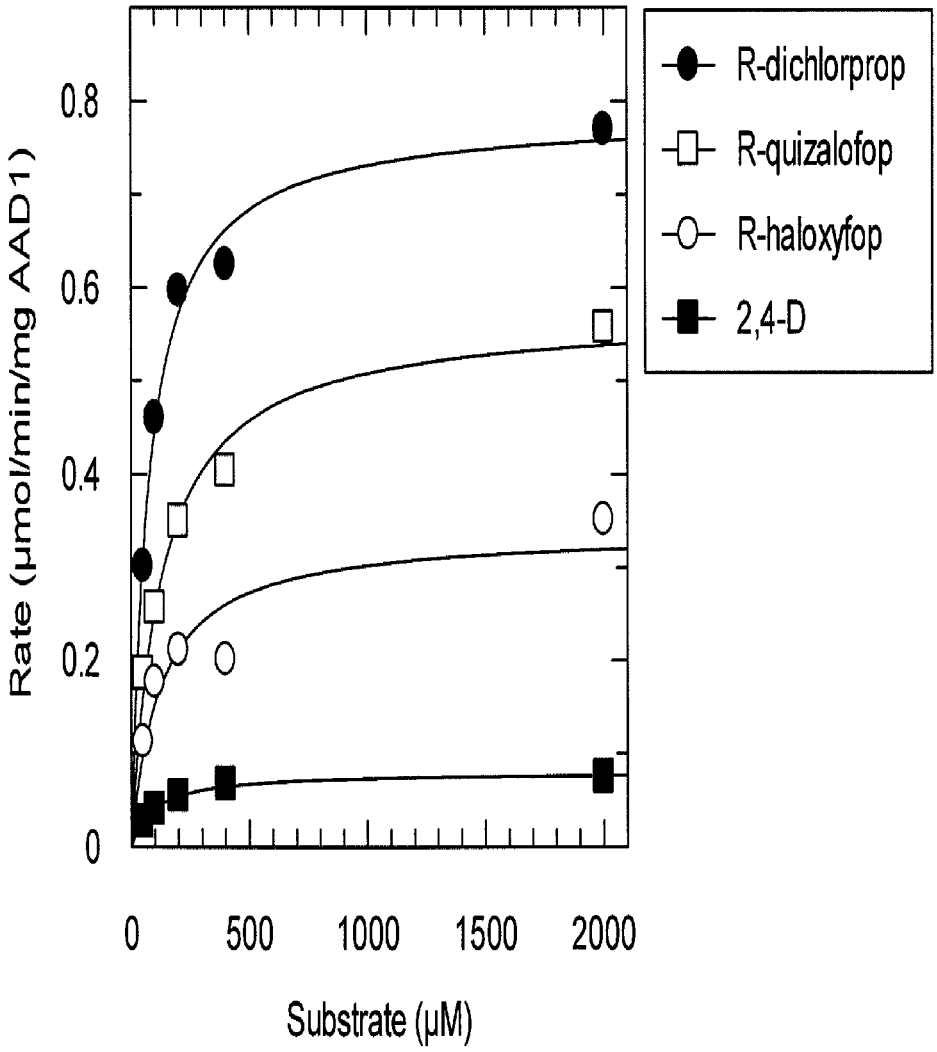

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

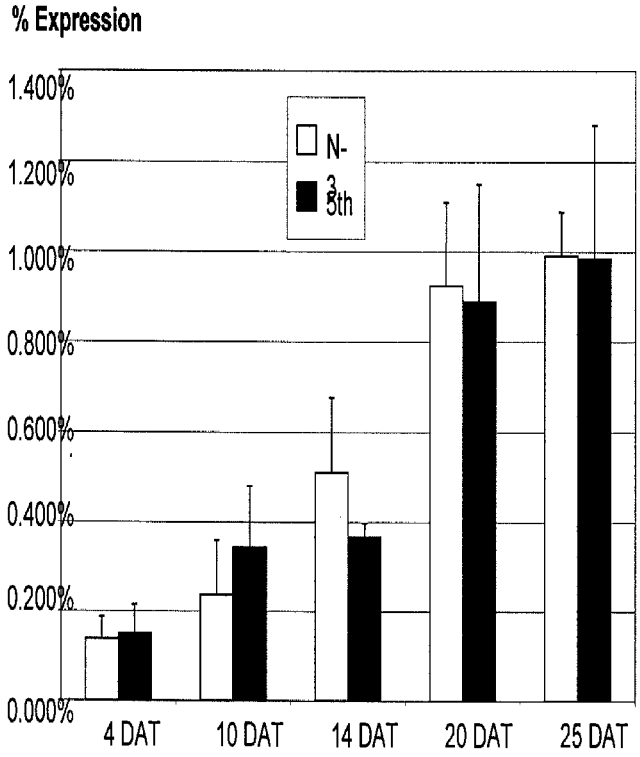

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

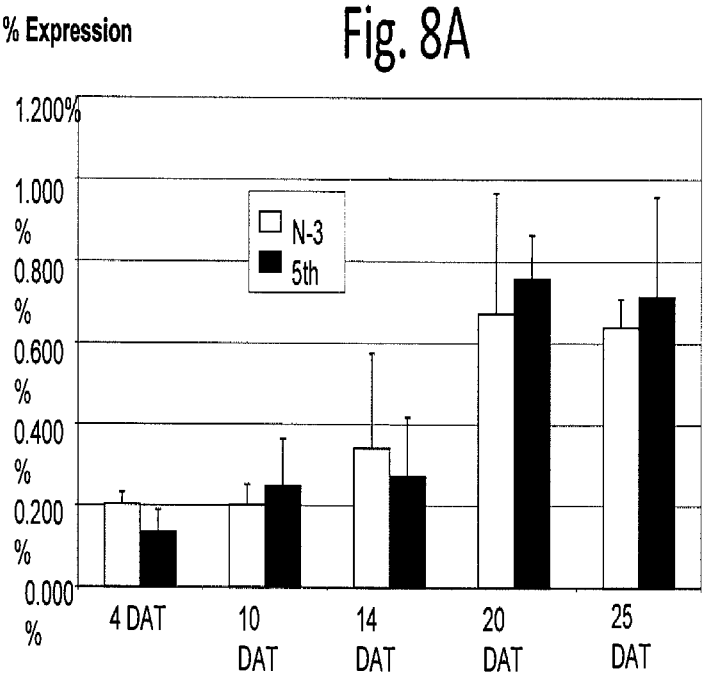

% Expression
1.200%
1.000 %
0.800 %
0.600 %
0.400 %
0.200 %
0.000 %
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

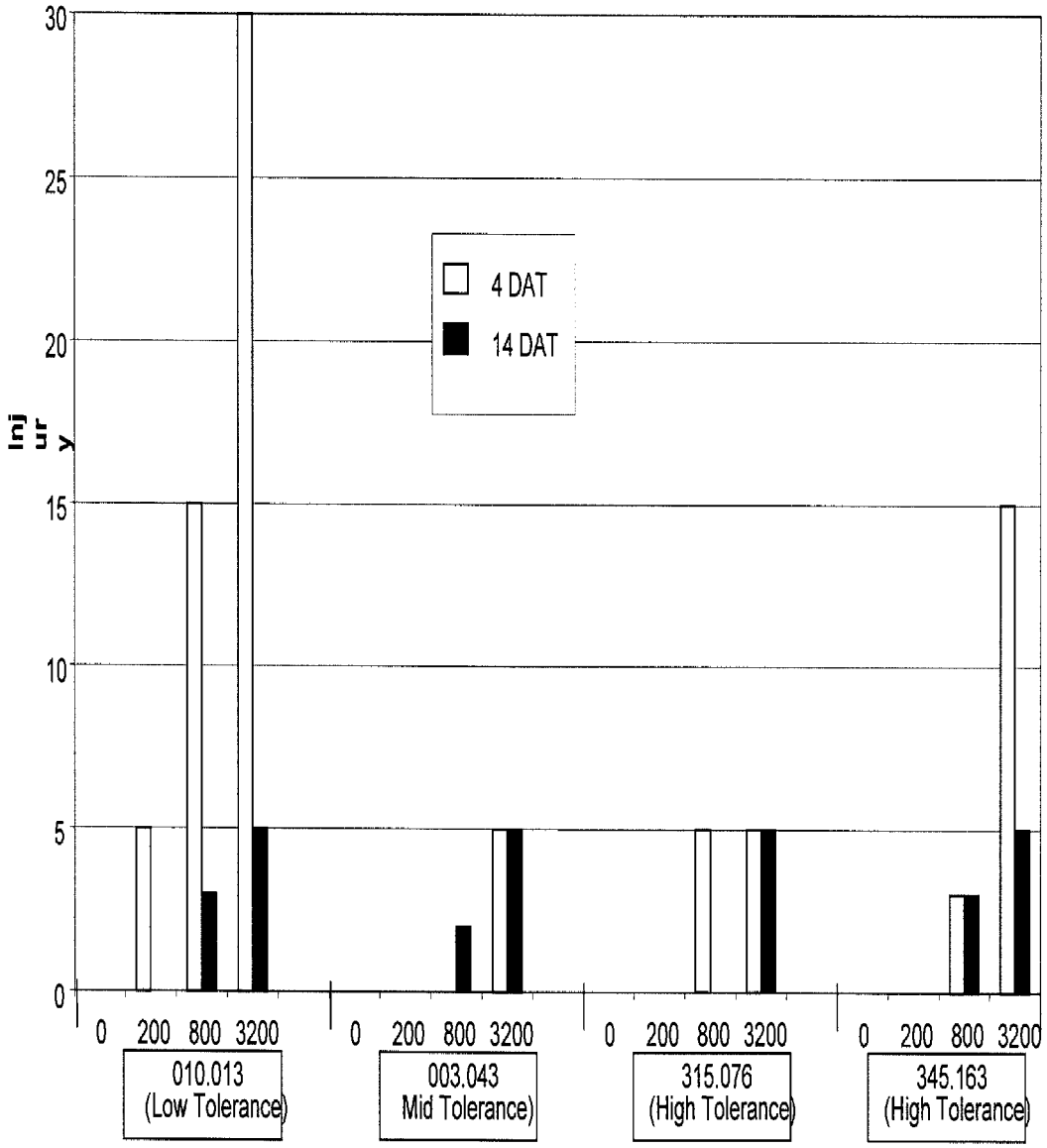

30
25
20
15
10
5
0
Inj
ur
y
4 DAT
14 DAT
0 200 800 3200
010.013
(Low Tolerance)
0 200 800 3200
003.043
Mid Tolerance)
0 200 800 3200
315.076
(High Tolerance)
0 200 800 3200
345.163
(High Tolerance)

Fig. 9A

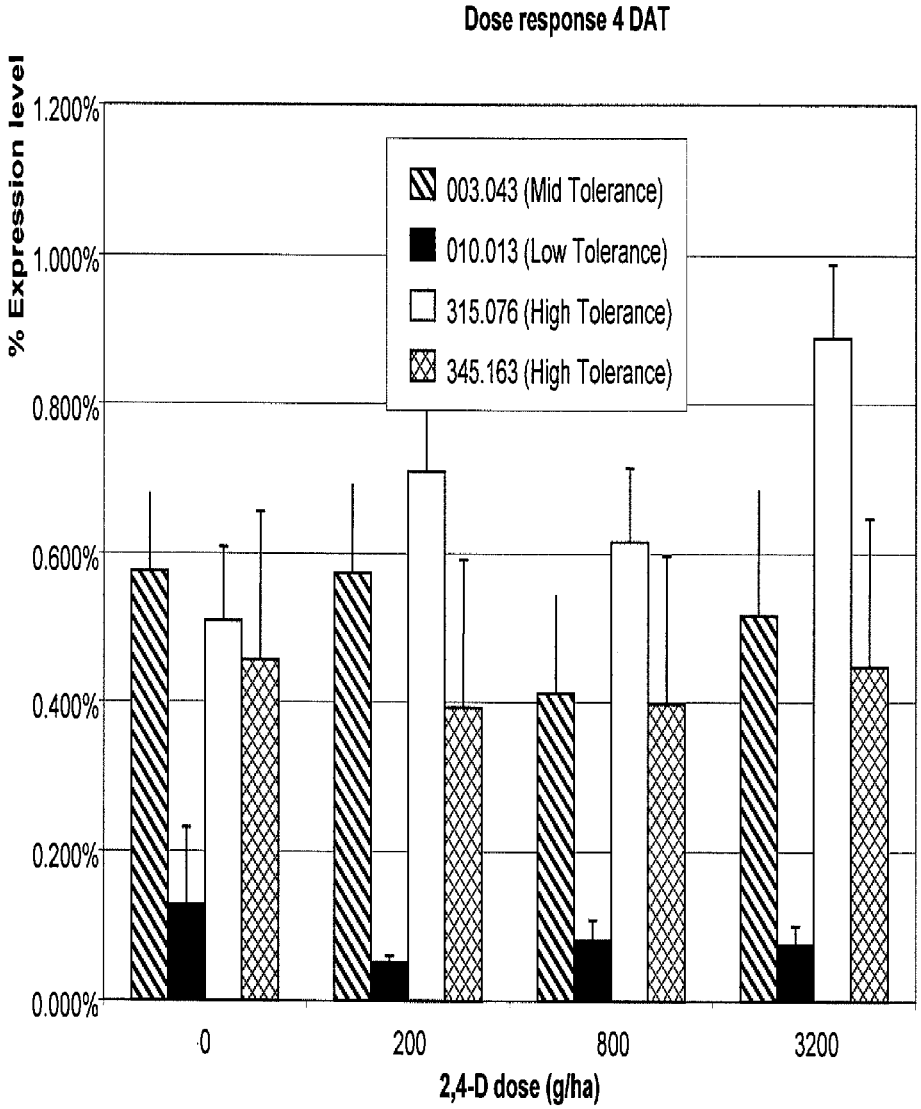

Dose response 4 DAT
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

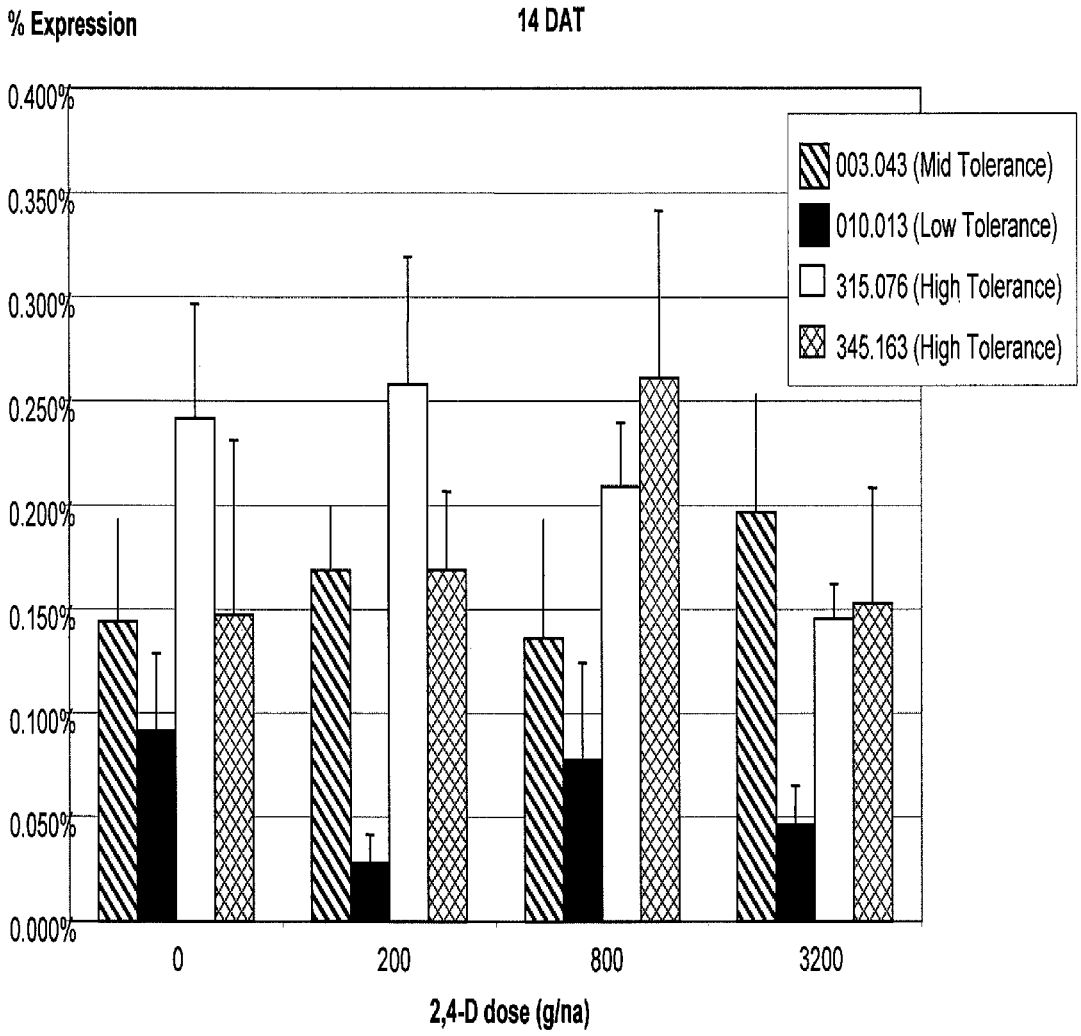

% Expression
14 DAT
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

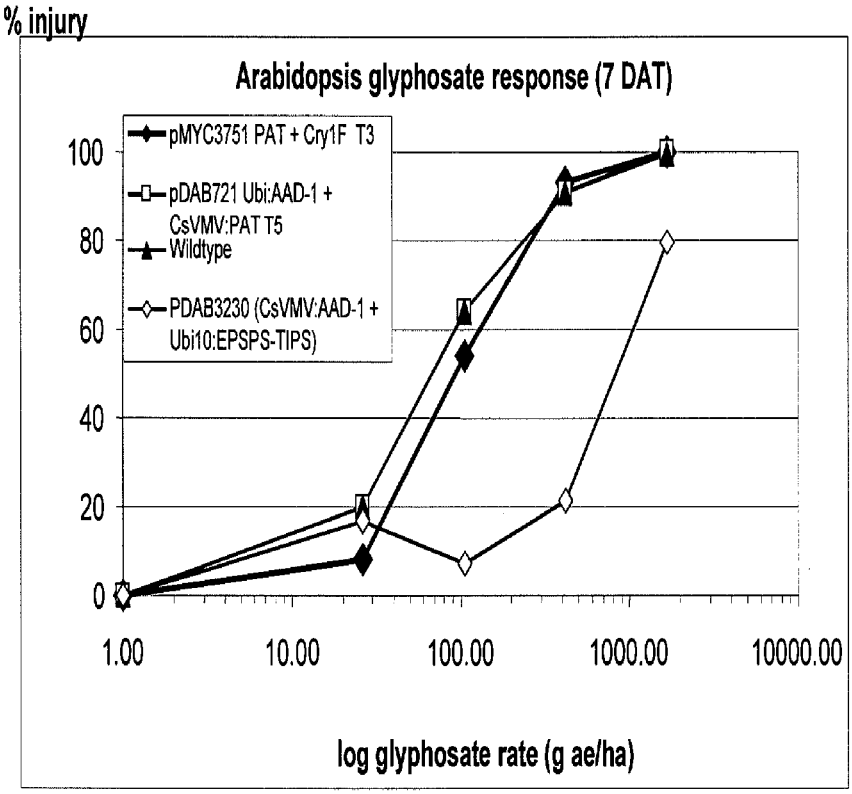

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

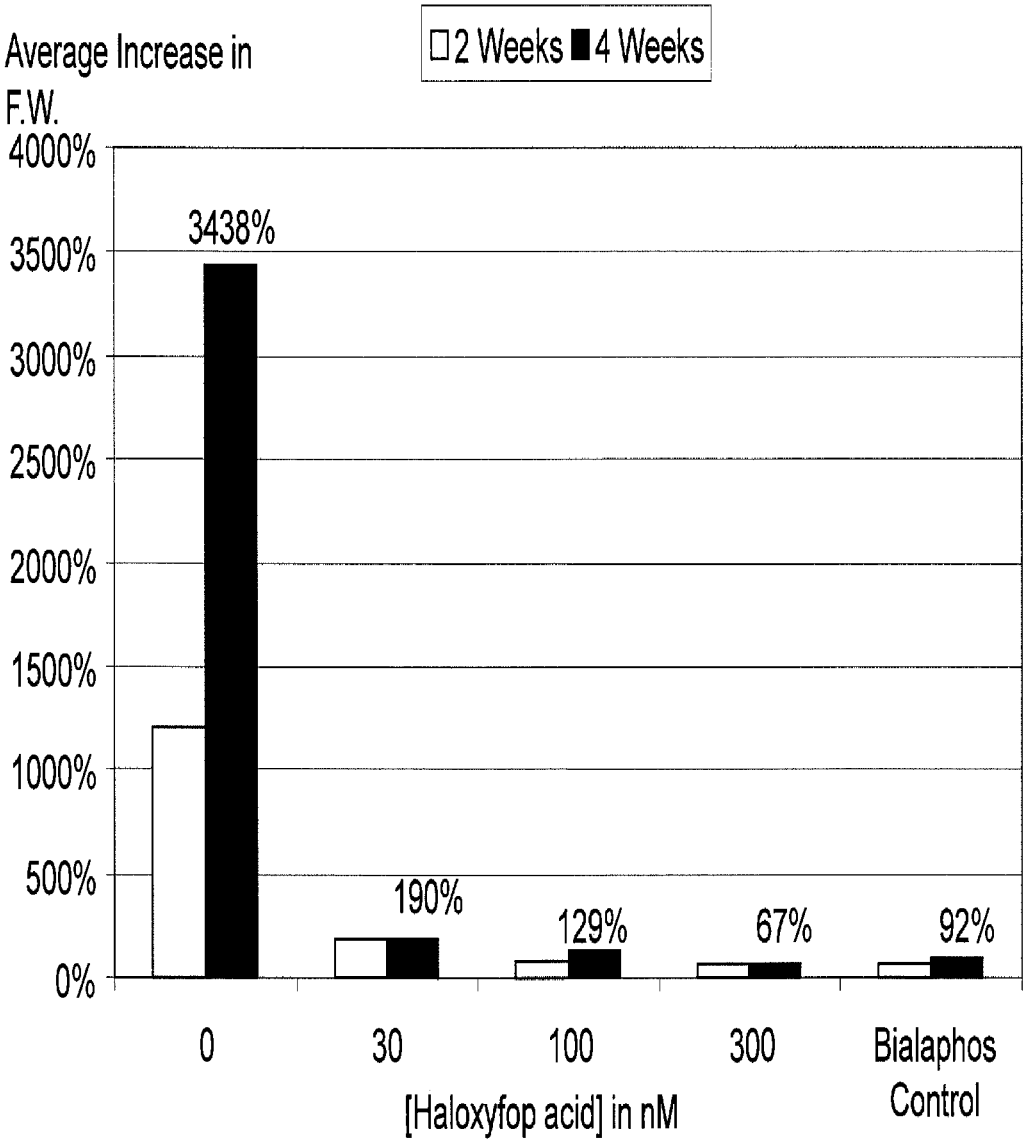

Average Increase in F.W.
2 Weeks
4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

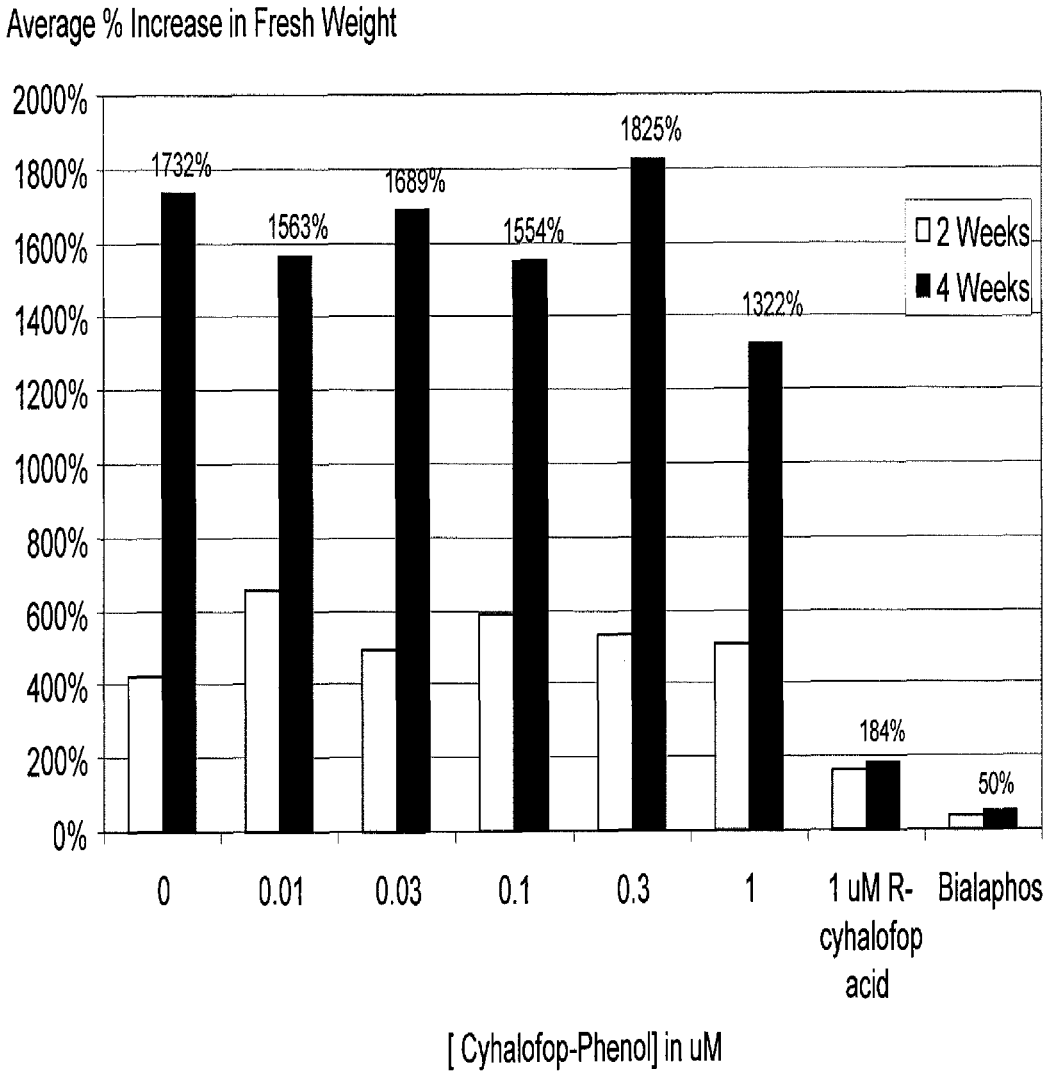

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
2 Weeks
4 Weeks
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

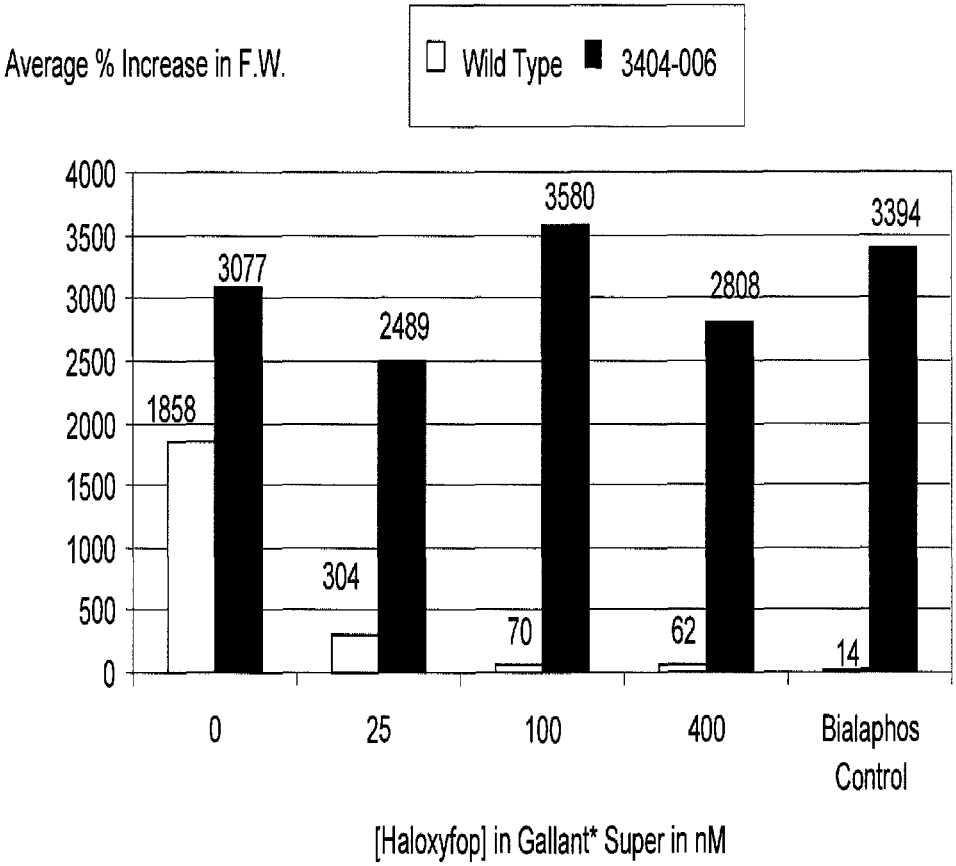

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
1858
3077
304
2489
70
3580
62
2808
14
3394
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant* Super in nM

Fig. 13

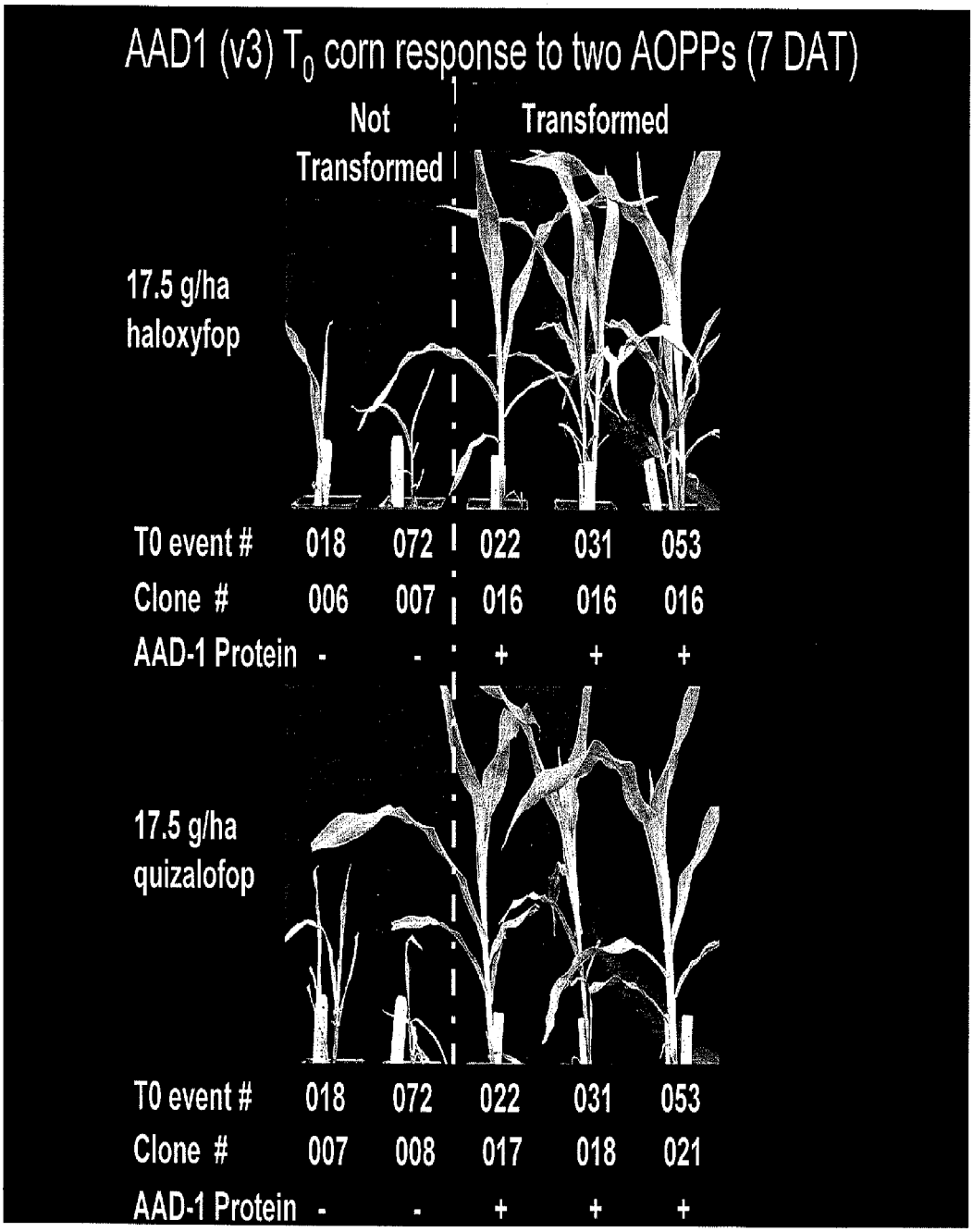

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

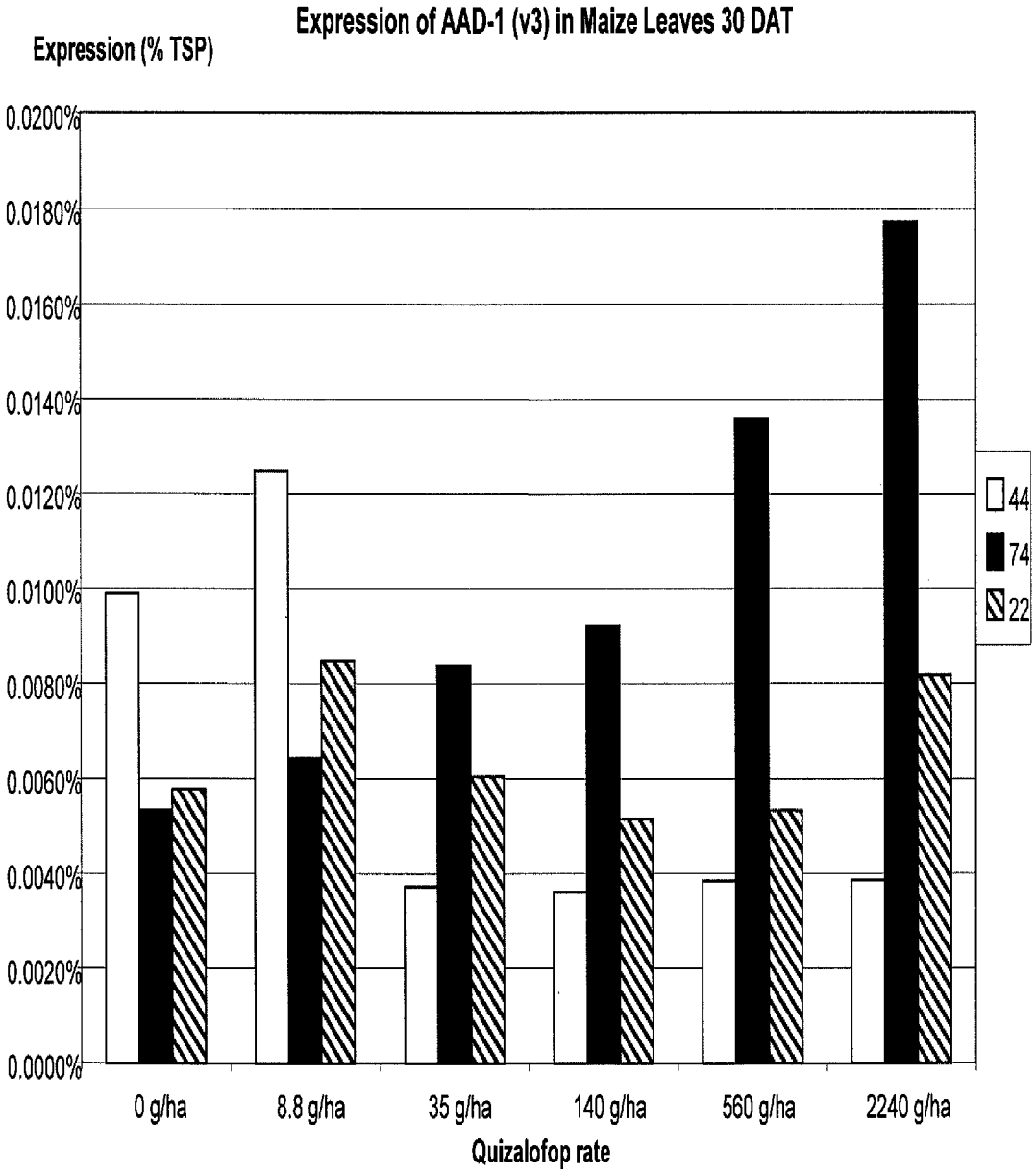

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
44
74
22
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate

Fig. 15


Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK-93
(untransformed)
Event 3
(AAD-1 transformed)

Fig. 16

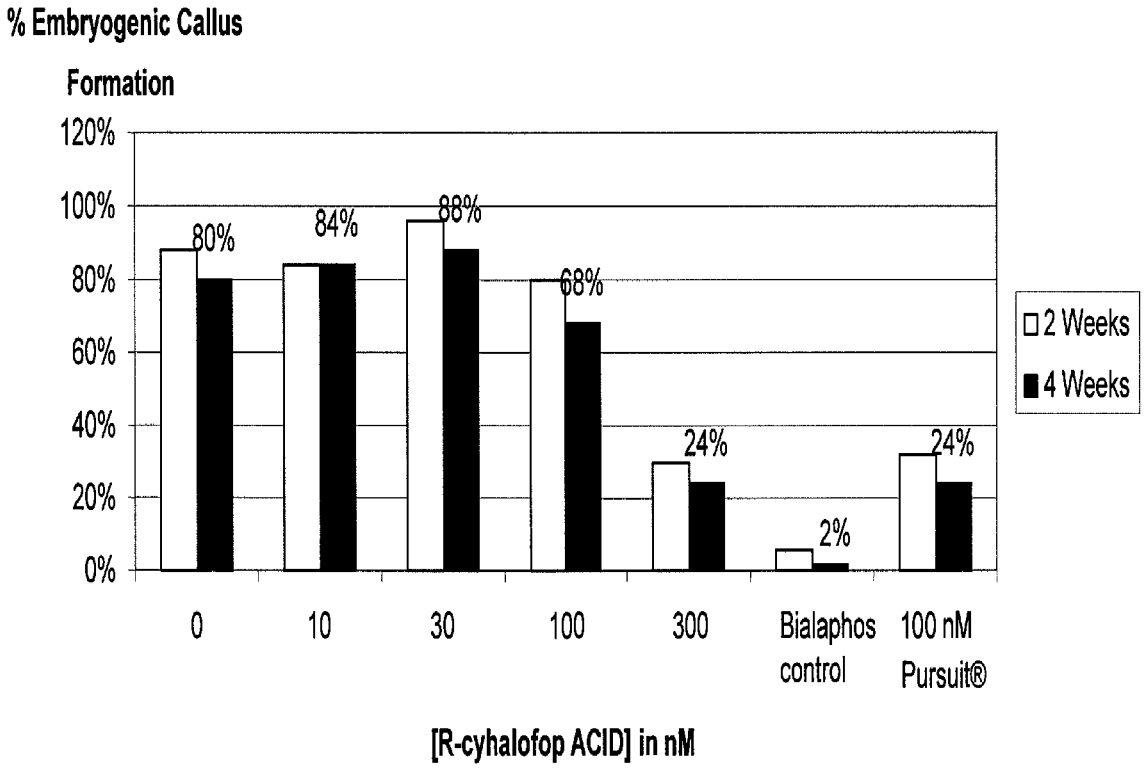
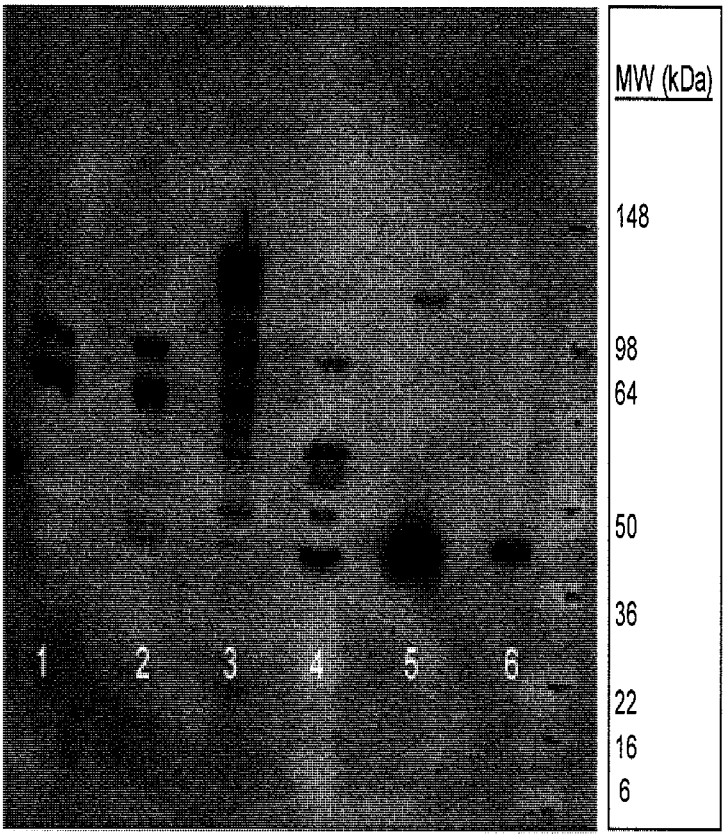

% Embryogenic Callus
Formation
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
2 Weeks
4 Weeks
[R-cyhalofop ACID] in nM

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

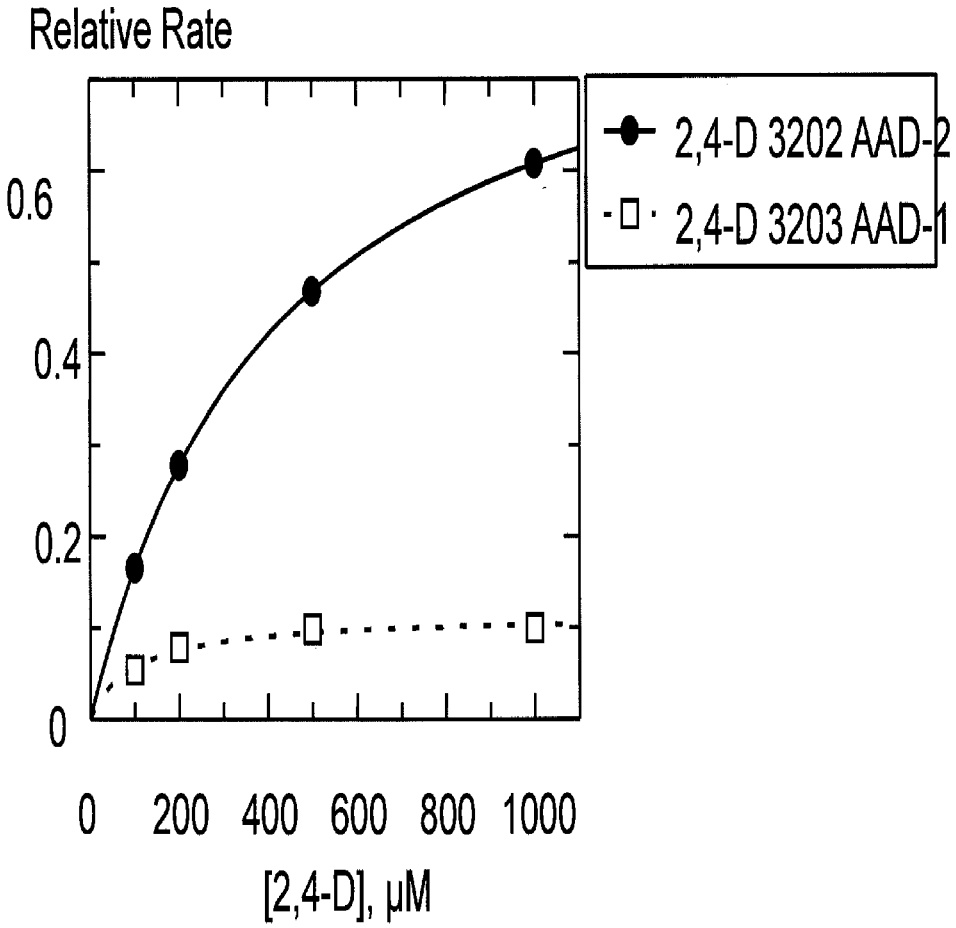

Relative Rate
0.6
0.4
0.2
0
0
200
400
600
800
1000
[2,4-D], µM
2,4-D 3202 AAD-2
2,4-D 3203 AAD-1

Fig. 19

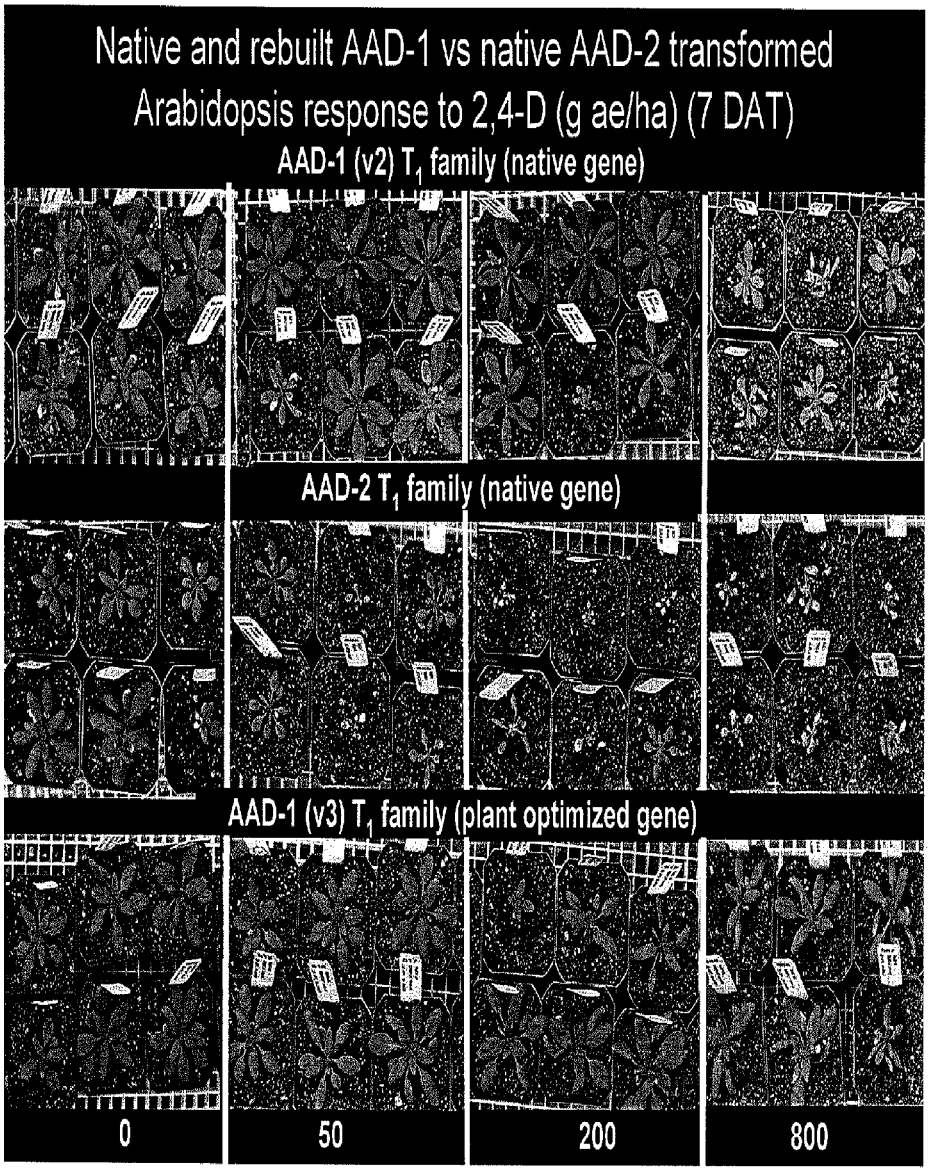

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

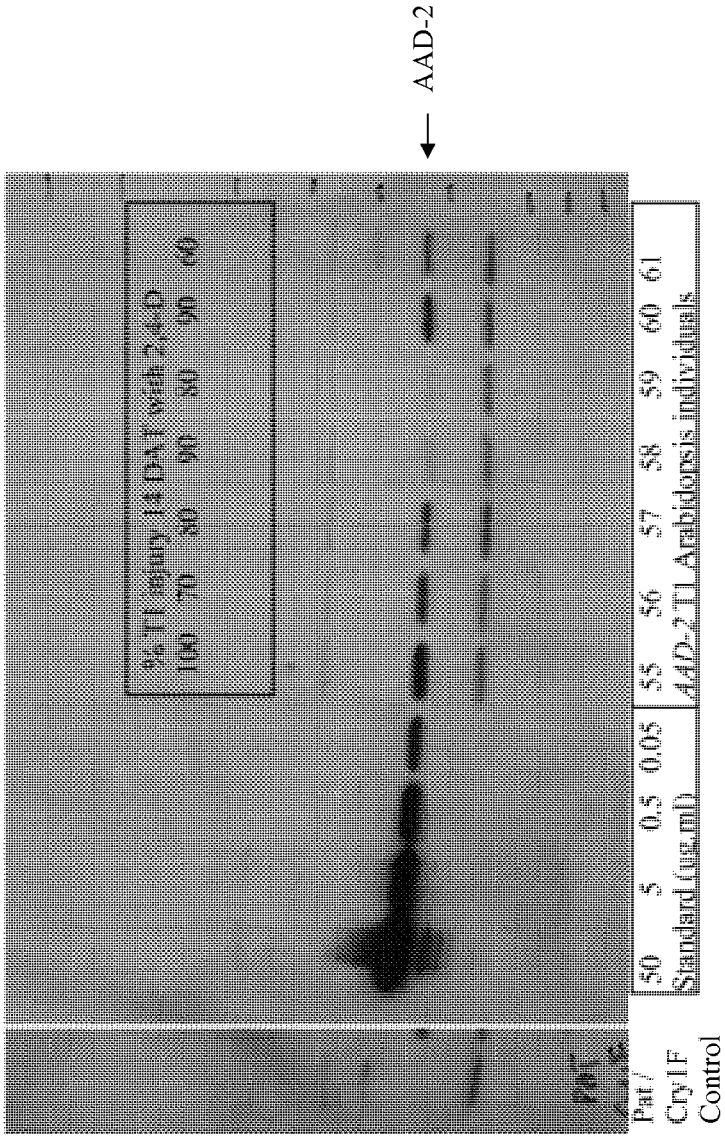

AAD-2
Pat / Cry1F Control
50 5 0.5 0.05
Standard (ug/mL)
55 56 57 58 59 60 61
AAD-2 T1 Arabidopsis individuals
% T1 injury 14 DAT with 2,4-D
100 70 60 50 40 30 20 10 0

Fig. 21

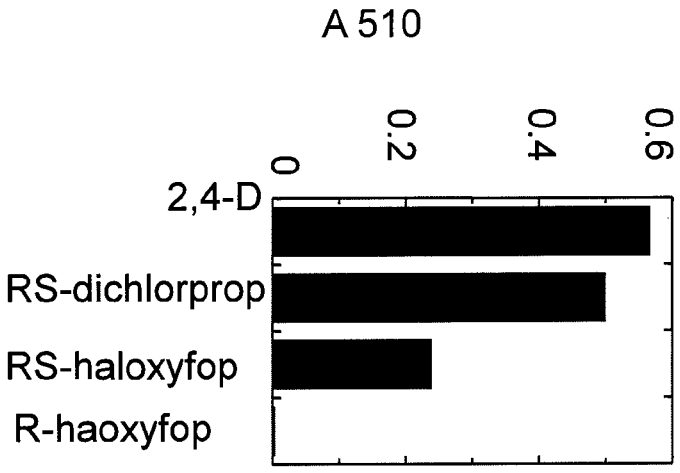


FIG. 22

HERBICIDE RESISTANCE GENES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation application of U.S. Ser. No. 14/820,893, filed Aug. 7, 2015, which is a continuation application of U.S. Ser. No. 12/951,813, filed Nov. 22, 2010, now patented as U.S. Pat. No. 9,127,289, which is a continuation of U.S. Ser. No. 11/587,893, filed May 22, 2008, now patented as U.S. Pat. No. 7,838,733, which is a national stage entry of PCT/US2005/014737, filed May 2, 2005, which claims the benefit of U.S. Provisional Application Ser. No. 60/567,052, filed Apr. 30, 2004, which are hereby incorporated by reference in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

BACKGROUND OF THE INVENTION

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.

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.

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.

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.

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.

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).

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.

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).

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.

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. Pat. 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. Pat. No. 5,608,147).

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.

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).

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.

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

BRIEF SUMMARY OF THE INVENTION

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.

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).

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.

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.

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

FIG. **1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

FIG. **2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

FIG. **3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

FIG. **4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

FIG. **5** shows 2,4-dichlorophenol production by recombinant AAD-1.

FIGS. **6**A and **6**B show phenol production by recombinant AAD-1 from various herbicide substrates.

FIG. **7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

FIGS. **8**A and **8**B 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.

FIGS. **9**A, **9**B, and **9**C 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.

FIG. **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.

FIG. **11** shows dose response of callused maize suspensions to R-haloxyfop.

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

FIG. **13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

FIG. **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.

FIG. **15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

FIG. **16** shows AAD-1 (v3)-transformed corn tolerant to 8× field rates of quizalofop (Assure II) under field conditions.

FIG. **17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

FIG. **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.

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

FIG. **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.

FIG. **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 AA D-2-transformed and Pat/Cry1F-transformed samples.

FIG. **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

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

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

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

SEQ ID NO:4 is the nucleic acid sequence of the native AAD-1 gene with internal NotI restriction site removed. This gene is designated AAD-1 (v2). DNA sequencing confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

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.

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).

SEQ ID NO:8 is another PCR primer ("BstEII/Del NotI") that was used with the "3' SacI" primer.

SEQ ID NO:9 is the native amino acid sequence encoded by the AAD-1 (v1) gene from *Sphingobium herbicidovorans*.

SEQ ID NO:10 is the amino acid sequence encoded by the AAD-1 (v2) DNA sequence of SEQ ID NO:4.

SEQ ID NO:11 is the amino acid sequence encoded by the AAD-1 (v3) plant-optimized DNA sequence of SEQ ID NO:5.

SEQ ID NO:12 is the DNA sequence of the native AAD-2 (v1) gene.

SEQ ID NO:13 is the amino acid sequence of the AAD-2 (v1) protein.

SEQ ID NO:14 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:15 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:16 is the M13 forward primer.

SEQ ID NO:17 is the M13 reverse primer.

SEQ ID NO:18 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:19 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:20 is the native soybean EPSPS protein.

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).

SEQ ID NO:22 is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

SEQ ID NO:23 is primer Pat 5-3.

SEQ ID NO:24 is primer Pat 3-3.

SEQ ID NO:25 is forward primer AAD-1 PTU.

SEQ ID NO:26 is reverse primer AAD-1 PTU.

SEQ ID NO:27 is the forward primer for the Coding Region PCR AAD-1.

SEQ ID NO:28 is the reverse primer for the Coding Region PCR AAD-1.

SEQ ID NO:29 is the AAD-2 (v2) nucleotide (plant optimized).

SEQ ID NO:30 is the translated AAD-2 (v2) protein sequence.

SEQ ID NO:31 is the Southern fragment PCR AAD-1 forward primer.

SEQ ID NO:32 is the Southern fragment PCR AAD-1 reverse primer.

DETAILED DESCRIPTION OF THE INVENTION

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.

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

TABLE 1

Commercially available phenoxy auxins.

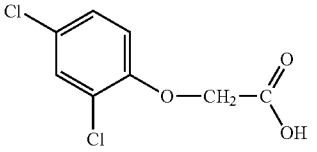
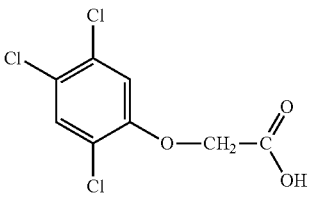
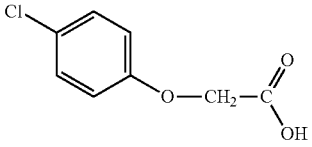
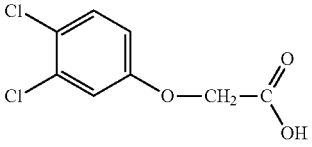
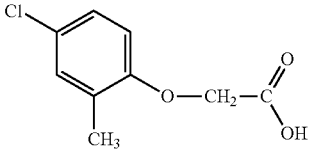
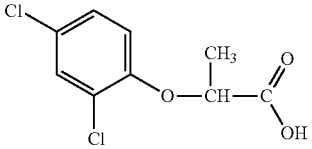
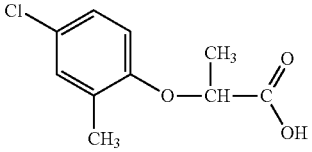
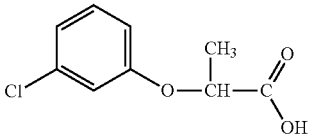
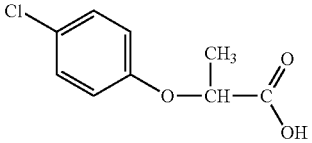
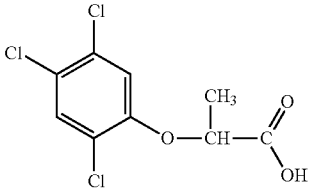
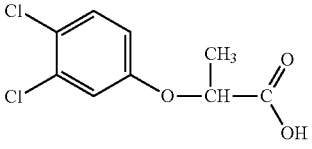
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	
2,4,5-T	93-76-5	25-4000	25-4000	

TABLE 1-continued

Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
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	
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	

An additional benefit of the AAD-1 gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). See Table 2. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing AAD-1, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, *sorghum*, warm and cool-season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

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.

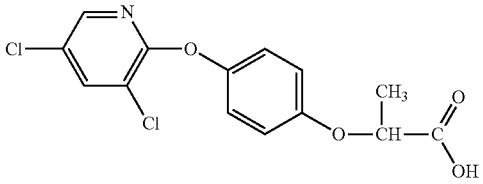
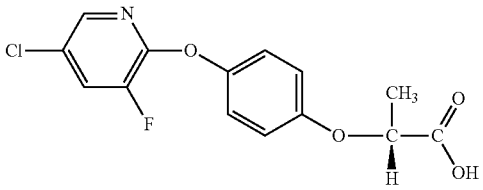
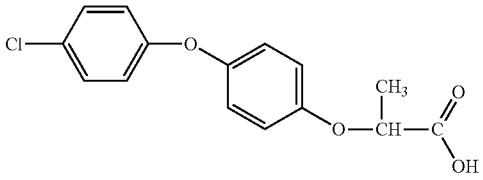
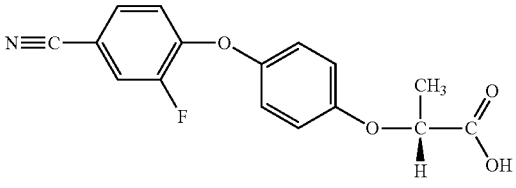
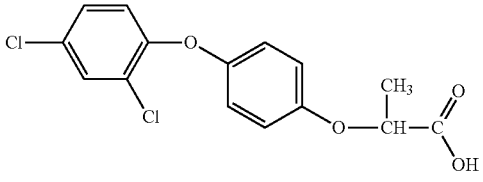
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Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

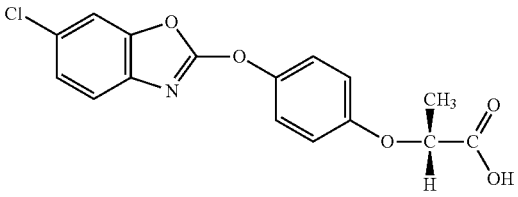
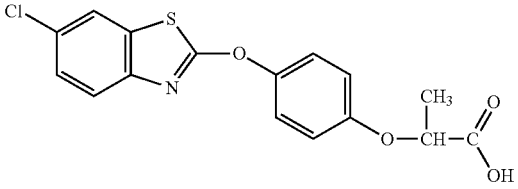
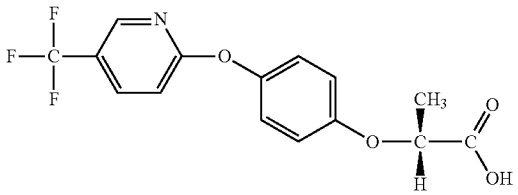
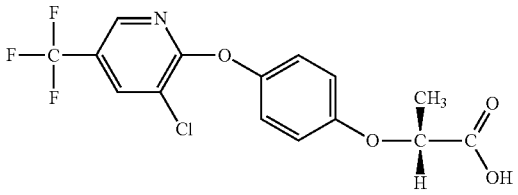
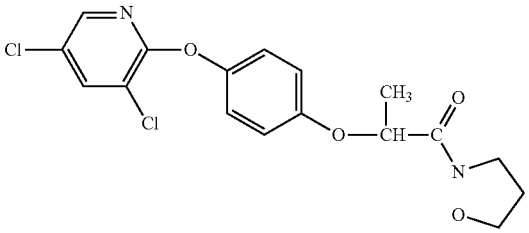
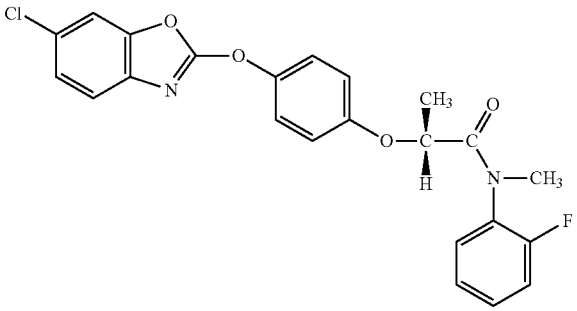
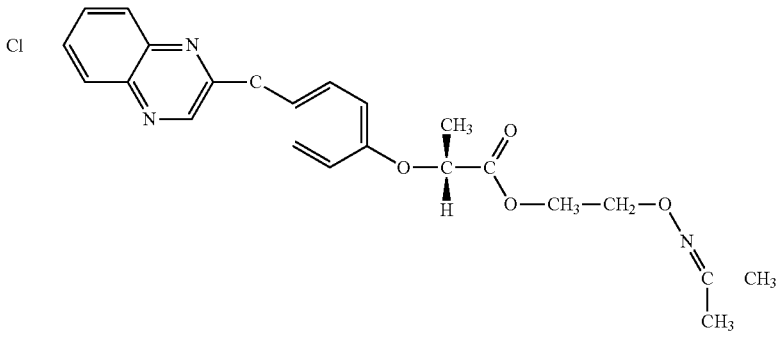
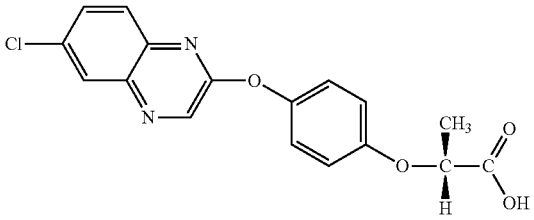
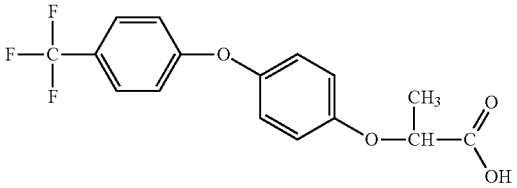
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenoxaprop	66441-23-4	10-2000	20-200	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	
Isoxapyrifop	87757-18-4	10-2000	30-240	
Metamifop	256412-89-2	10-2000	35-280	

TABLE 2-continued

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
Propaquizafop	111479-05-1	10-2000	30-240	
Quizalofop	76578-14-8	10-2000	20-240	
Trifop	58597-74-4	10-2000	10-2000	

A single gene (AAD-1) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, AAD-1 can provide protection in planta to AOPP herbicides where natural tolerance also was not sufficient to allow selectivity. Plants containing AAD-1 alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combinations of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

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).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-1 stack

could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

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.

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 TT 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.

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, carfentrazonc, sulfentrazonc, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

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.

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.

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. As disclosed herein a peptide of SEQ ID NO: 9 is provided that is capable of degrading 2,4-D, and AOPP herbicides. Mapping the α-ketoglutarate-dependent dioxygenase motif disclosed in the previous paragraph to the sequence of SEQ ID NO: 9 reveals the AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids.

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.

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.

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.

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.

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.

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. Pat. 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.

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).

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).

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 1 mM 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).

TABLE 3

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

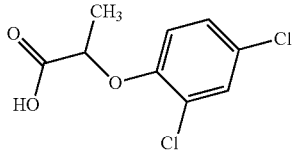
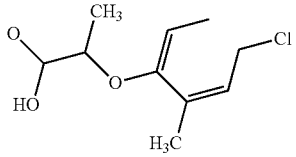
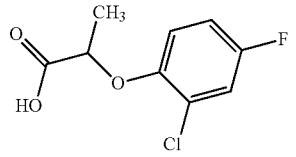
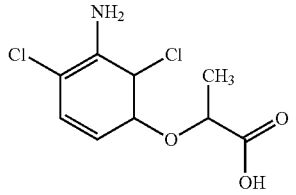
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676

TABLE 3-continued

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

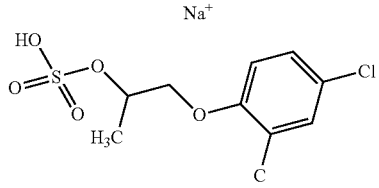
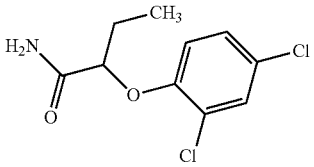
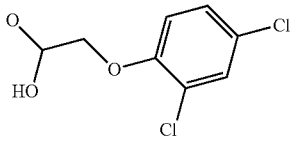
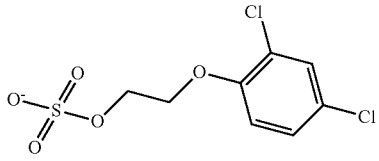
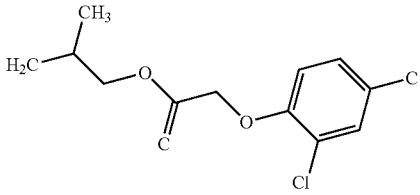
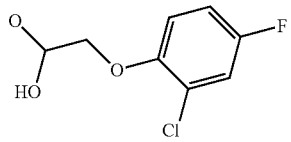
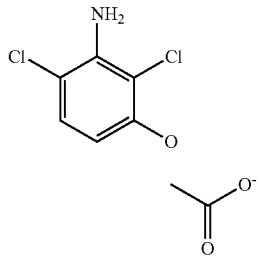
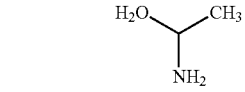
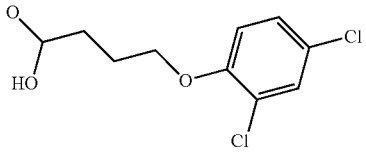
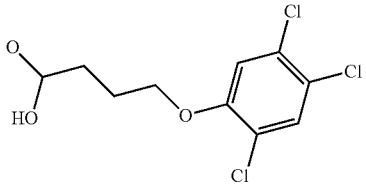
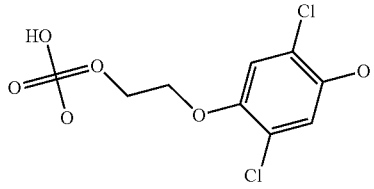
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058
	11263526		0.004	0.069

TABLE 3-continued

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

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	178577		0.003	0.021
				
Na^+	178587		0.003	0.02
				
Na^+	188527		0.003	0.036
				

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.

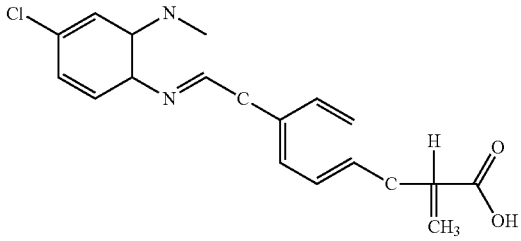
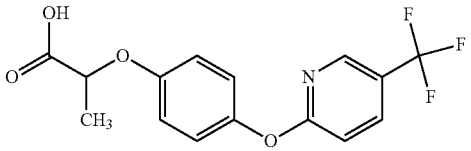
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17

TABLE 4-continued

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

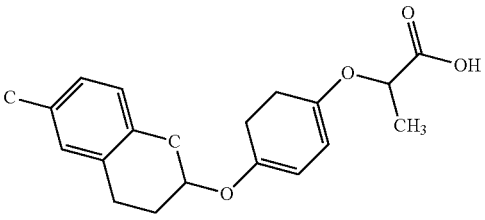
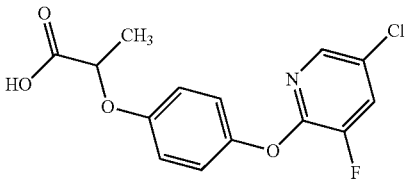
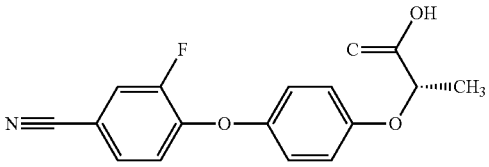
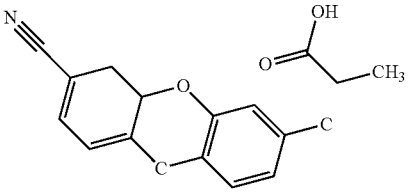
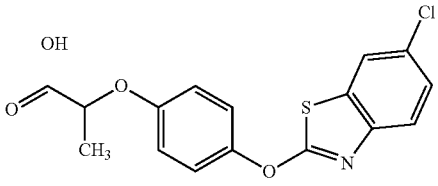
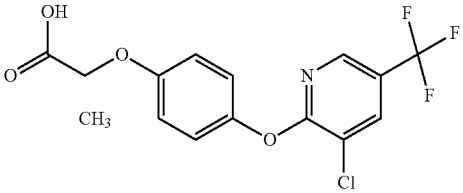
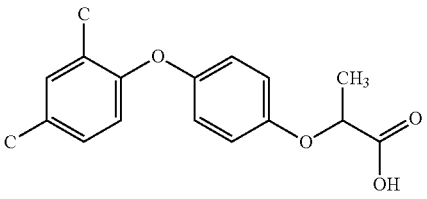
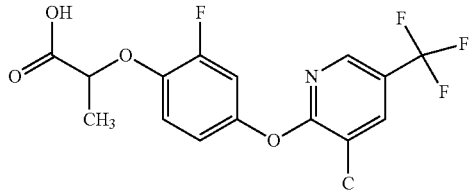
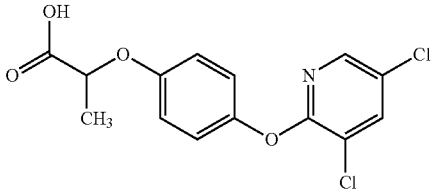
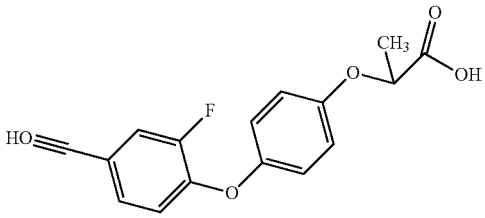
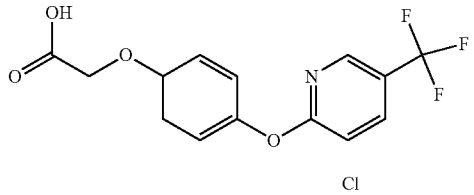
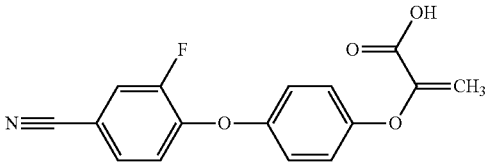
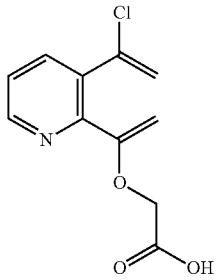
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	11044492	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinofop	0.295	1.98
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63
	460511	(R,S)-diclofop	0.155	1.663

TABLE 4-continued

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

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.).

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, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2×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 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×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 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×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.

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.

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.

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 6×SSPE, 5×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.

Washes can typically be carried out as follows:

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

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

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C. below the melting temperature (Tm) of the hybrid in 6×SSPE, 5×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).

Washes can typically be out as follows:

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

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

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 2×SSPE, room temperature

Low: 1 or 2×SSPE, 42° C.

Moderate: 0.2× or 1×SSPE, 65° C.

High: 0.1×SSPE, 65° C.

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.

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. Pat. 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.

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.

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.

In addition, U.S. Pat. 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.

"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.

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.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases accord-

ing to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

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., Hofte et al. (1989), and Adang et al. (1985)). As used herein, the term "protein" can include functionally active truncations.

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.

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

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.

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

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.

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."

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."

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.

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.

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.

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.

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.

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. Pat. No. 5,135,867.

Vectors comprising an AAD-1 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley et al. (1986); and An et al. (1985).

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.

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.

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.

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.

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. No. 4,945,050 to Cornell and U.S. Pat. No. 5,141,131 to Dow-Elanco, 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; U.S. Pat. No. 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 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. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

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.

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

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.

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.

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.

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.

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, 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.

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.

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. No. 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.

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.

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

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.

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.

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.

A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward:

[(SEQ ID NO: 1)

5' TCT AGA AGG AGA TAT ACC ATG CAT GCT GCA CTG

TCC CCC CTC TCC CAG CG 3'

(added Xba I restriction site and Ribosome Binding Site (RBS))] and Reverse:

[(SEQ ID NO: 2)

5' CTC GAG TTA CTA GCG CGC CGG GCG CAC GCC ACC

GAC CG 3'

(added extra stop codon and Xho I site)].

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 ~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.

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).

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.

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.

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.

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).

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.

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.

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) 250×4.6 mm (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.

2.2—Plate Test Bioassays for Herbicide Degradation.

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.

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.

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 $NaMoO_4.2H_2O$, 10 μM NaCl, 10 nM $CoCl_2.H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60×15-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.

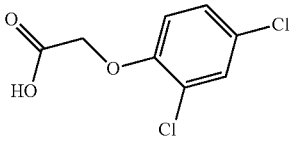
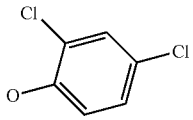
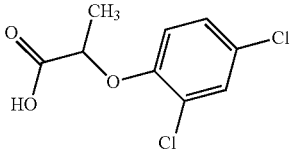
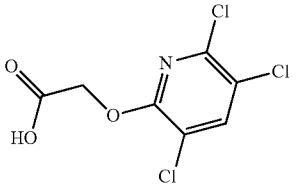
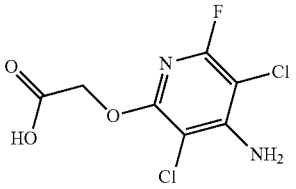
For herbicide-enzyme test solutions, the maximal concentration was determined based on the nominal concentration before any subsequent enzymatic degradation would occur. Seeds were evenly spread by adding 3 ml of melted PTM of the same composition, swirling, and allowing to solidify. Plates were sealed 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 bent-

grass plants, respectively, average mean length (percent of untreated control) vs. nominal herbicide concentration and GR_{50} determined.

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

TABLE 6

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

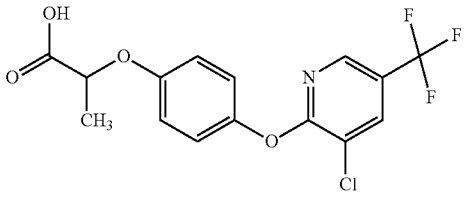
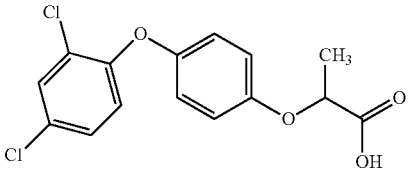
	GR_{50} (nM)				
Chemical tested	Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
2,4-D	22	17	267	16	
DCP	>1000	nd	nd	nd	
Dichlorprop	nd	30	1000	33	
Triclopyr	255	1000	1000	1	
Fluroxypyr	2200	2250	1825	<1	

* 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 vetor 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	GR50 (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.

2.3—HPLC Results.

From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see FIG. **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.

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 (FIG. **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.

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).

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 (FIG. **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.

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 (FIG. **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.

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 (FIG. **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

3.1—AAD-1 (v1) Assay.

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 FIG. **4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color yields to that of 2,4-dichlorophenol and so were readily detected. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol and the haloxyfop phenol was linear and proportional to the concentration of phenol in the assay up to ~500 μM. A calibration curve performed under standard assay conditions (160 μl final assay volume) indicated that an absorbance at 510 nm of 1.0 was obtained from 172 μM phenol.

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.

3.2—Extraction.

Activity of recombinant AAD-1 (v1) expressed in *Escherichia coli.*

E. coli cell pellets were resuspended in 0.1 M Tris, pH 7.4+1 mg/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.

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 Op using 1 mM 2,4-D and is shown in FIG. **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.

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. FIG. **6**A 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.

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 FIG. **6**B. 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—Additional substrates of AAD-1 (v1).

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).

Table 4 shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop

rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

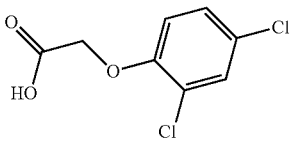
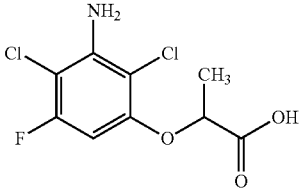
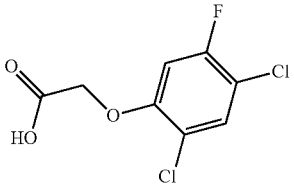
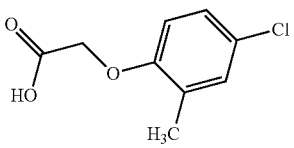
Substrates X11115427, X124987 and MCPA were tested at 1 mM using 27 μg crude recombinant AAD-1 (v1) per assay. All three compounds were substrates for AAD-1 (v1) but with different relative effectiveness (Table 8). X11115427 was slightly better as a substrate than 2,4-D (125% of the 2,4-D rate) in contrast 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.

Table 8:

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

TABLE 8

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

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

4.2—Kinetic Characterization.

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^{e+}; 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 FIG. **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).

Table 9:

Kinetic Constants for AAD-1 (v1) Substrates. Kinetic constants were derived from the data in FIG. **7** using Graft fitting to the Michaelis-Menten equation.

TABLE 9

Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (μM) ± SE	V_{max} (μmol min^{-1} mg^{-1} AAD1) ± SE	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
R-dichlorprop	75 ± 10	0.79 ± 0.03	26.1	348
R-quizalofop	125 ± 20	0.57 ± 0.03	18.9	151

TABLE 9-continued

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-haloxyfop	120 ± 54	0.34 ± 0.04	11.2	94
2,4-D	96 ± 8	0.57 ± 0.00	2.7	28

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.

4.3—Additional substrates for AAD-1 (v1).

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

5.1—Background.

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.

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.

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 MacVector™ program (Accelerys, San Diego, Calif.). 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.aNumber of genes in class given in parentheses.
.sup.bStandard deviations given in parentheses.
.sup.c Combined groups mean ignored in mean calculation

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.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 11. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first,

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

TABLE 11

Preferred amino acid codons for proteins expressed in maize

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

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.

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. Pat. No. 5,380,831.

5.2—Rebuild Analysis.

Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native AAD-1 (v1) coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5) was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and Table 12 shows the differences in codon compositions of the native and plant-optimized sequences.

TABLE 12

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

Amino Acid	Codon	Native Gene #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %	Amino Acid	Codon	Native Gene #		Plant Opt Gene #	Plant Opt Gene %
ALA (A)	GCA	2	9.1	5	22	LEU (L)	CTA	0	0.0	0	0
22	GCC	10	45.5	8	35	22	CTC	5	22.7	7	32
	GCG	9	40.9	0	0		CTG	16	72.7	0	0
	GCT	1	4.5	10	43		CTT	0	0.0	8	36
ARG (R)	AGA	0	0.0	7	30		TTA	0	0.0	0	0
23	AGG	0	0.0	7	30		TTG	1	4.5	7	32
	CGA	0	0.0	0	0	LYS (K) 7	AAA	1	14.3	2	29
	CGC	16	69.6	5	22		AAG	6	85.7	5	71
	CGG	4	17.4	0	0	MET (M)	ATG	8	**100**	8	**100**
	CGT	3	13.0	4	17	PHE (F)	TTC	10	76.9	9	69
ASN (N) 8	AAC	8	100.0	4	50	13	TTT	3	23.1	4	31
	AAT	0	0.0	4	50	PRO (P)	CCA	1	5.9	8	47
ASP (D)	GAC	15	78.9	10	53	17	CCC	9	52.9	1	6
19	GAT	4	21.1	9	47		CCG	7	41.2	0	0
CYS (C) 3	TGC	3	100.0	2	67		CCT	0	0.0	8	47
	TGT	0	0.0	1	33	SER (S)	AGC	11	73.3	4	27

TABLE 12-continued

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 #	Native Gene %	Plant Opt Gene #	Plant Opt Gene %
END 1	TAA	0	0.0	0	0	15	AGT	0	0.0	0	0
	TAG	1	100.0	0	0		TCA	0	0.0	4	27
	TGA	0	0.0	1	100		TCC	2	13.3	3	20
GLN (Q)	CAA	0	0.0	7	54		TCG	2	13.3	0	0
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	23	ACC	16	69.6	9	39
GLY (G)	GGA	0	0.0	6	29		ACG	5	21.7	0	0
21	GGC	15	71.4	7	33		ACT	1	4.3	8	35
	GGG	3	14.3	2	10	TRP (W)	TGG	5	**100**	5	**100**
	GGT	3	14.3	6	29	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	28	GTC	11	39.3	8	29
12	ATC	12	100.0	5	42		GTG	15	53.6	10	36
	ATT	0	0.0	4	33		GTT	0	0.0	10	36
	Totals	148		149			Totals	148		148	

5.3—Completion of Binary Vectors.

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 1-Sac 1 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).

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).

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.

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.

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).

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.

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*.

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.

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 13. Columns D and H of Table 13 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 13. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

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

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

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 Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H 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

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.

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 Tex.).

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, Calif. 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.

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

TABLE 14

Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species* Transformed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	—	—	—
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	—	—
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—
3403		Cn, R	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3404		Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3415	1283	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3602	1421	Cn	AAD1 v3	ZmUbi1	—	RB7 Mar v2	—	—
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	—	—

pDAB #	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721	Erythromycin	—	pat	CsVMV	Agro binary
3230	Spectinomycin	—	AAD1 v3	CsVMV	Agro binary
3289	Spectinomycin	sacB	HptII	AtUbi3	Agro binary
3291	Spectinomycin	—	HptII	AtUbi3	Agro binary
3295	Spectinomycin	—	pat	AtUbi10	Aerosol beam
3297	Spectinomycin	—	pat	AtUbi10	Agro binary
3403	Ampicillin	—	Same as GOI		Whiskers/ Gun
3404	Ampicillin	—	pat	OsAct1	Whiskers
3415	Ampicillin	—	AHAS v3	OsAct1	Whiskers

TABLE 14-continued

Binary constructs used in transformation of various plant species.

3602	Spectinomycin	—	AHAS v3	OsAct1	Agro Superbinary
3757	Spectinomycin	—	pat	AtUbi11	Agro binary
3705	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
ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter
AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter
HptII = hygromycin phosphotransferase
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

Example 6

Transformation into *Arabidopsis* and Selection

6.1—*Arabidopsis thaliana* Growth Conditions.

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).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) 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.

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^2 sec under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2—*Agrobacterium* Transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/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, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

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: 2.4 kV, Pulse length: 5 msec.

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

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 Minis Bio Inc. (Madison, Wis.) per manufacturer's instructions at 0.5× 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.

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. 8700×g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: ½× Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044 μM 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.

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 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), 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.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) 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).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-1f 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.

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, Del.) 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 3200 g ae/ha).

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.

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.

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.

6.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3) (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15). A small lot of AAD-1 (v2) (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

TABLE 15

Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

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

[1]Glufosinate selection scheme: 280 g 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

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 (6× 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.

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

TABLE 16

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

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

TABLE 16-continued

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	
					Std
Averages	<20%	20-40%	>40%	Ave	Dev
200 g ae/ha 2,4-D	11	4	1	14.0	25.9
800 g ae/ha 2,4-D	11	4	1	14.7	26.1
Wildtype					
Untreated control-buffer	11	0	0	0.0	0.0
50 g ae/ha 2,4-D	0	0	15	90.0	0.0
200 g ae/ha 2,4-D	0	0	15	95.1	0.5
800 g ae/ha 2,4-D	0	0	15	100.0	0.0
PAT/Cry1F (transformed control)					
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

Table 17 shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400-1,600 g ae/ha (Table 18). Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ac/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.

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

T1 *Arabidopsis* response to a range of dichlroprop rates applied postemergence.

	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
AAD-1 v3						
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						
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						
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

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.

	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
AAD-1 v3						
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						
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						
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

6.6—AAD-1 (v3) as a Selectable Marker.

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 (50 g 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.

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.

6.7—Heritability.

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 ($P>0.05$).

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.

6.8—Additional Herbicide Resistance Attributable to AAD-1 in *Arabidopsis*.

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.

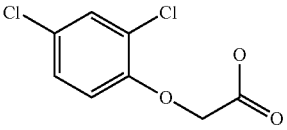
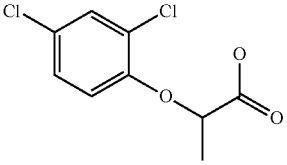
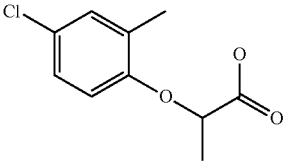
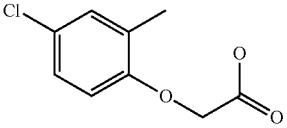
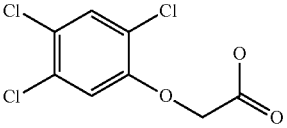
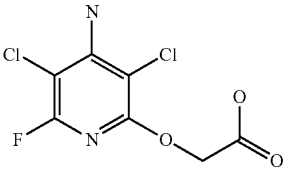
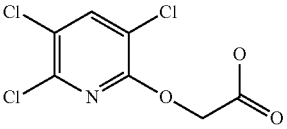
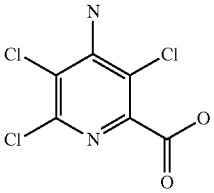
Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C.) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration 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, N.J.). 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).

Assessment of Growth Reduction.

The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in Table 19. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported in planta. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of AAD-1-transformed commercial crops or experimental plant species.

TABLE 19

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

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

6.9—Foliar Applications Herbicide Resistance in AAD-1 *Arabidopsis*.

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.

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.

Herbicide Treatment	Ave % Injury 14DAT: AAD1.01.315.076.T_4 homozygous AAD1 plants	Ave % Injury 14DAT: PatCry1f - Control
Phenoxypropionic auxins		
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		
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
Pyridyloxyacetic auxins		
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

6.10—Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

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. FIGS. **8**A and **8**B 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.

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 (FIG. **9**A). These plants also expressed high level of AAD-1 (v3), from 0.1 to 0.25% of (FIG. **9**B). 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 (FIG. **9**A), 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.

6.11—Molecular Analysis of AAD-1 (v3) *Arabidopsis*.

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 transfor-

mation unit containing PAT and AAD-1 (v3). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

For Southern analysis, a total of 1 μg of DNA was subjected to an overnight digest of Nsi 1 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× 100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of PAT. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

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.

6.12—*Arabidopsis* Transformed with Molecular Stack of AAD-1 (v3) and Glyphosate Resistance Gene.

T1 *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 FIG. **10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate+2,4-D treatments applied in tankmix as described in Example 21 and shown for AAD-1 (v3)-transformed corn in Example 8.

Example 7

WHISKERS-Mediated Transformation into Maize, and Use of AAD-1 (v3) as a Selectable Marker

7.1—Cloning of AAD-1 (v3).

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.

7.2—Callus/Suspension Initiation.

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.

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.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after

initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

7.3—Cryopreservation and Thawing of Suspensions.

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.

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 100×25 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.

7.4—Dose Response of Non-Transformed Tissue to Haloxyfop Acid.

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 60×20 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 FIG. **11**.

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 FIG. **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).

7.5—WHISKERS-Mediated Transformation Using Bialaphos Selection.

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

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.

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 60×20 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.

After 1 week, filter papers were transferred to 60×20 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).

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 100×15 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.

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 60×20 mm

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

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 μEm^2s^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 150×25-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.

7.6—WHISKERS-Mediated Transformation Using Haloxyfop Selection.

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.

After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60×20-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.

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.

7.8—Quantitative Demonstration of In Vitro Tolerance from AAD-1 (v3) Expressing Callus Events from Bialaphos Selection

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	+	+

TABLE 21-continued

Event	PAT Copy #	PCR for AAD-1 (v3) PTU	Callus Western
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	+	+

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 FIG. **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.

7.9—WHISKERS-Mediated Transformation Using Imazethapyr Selection.

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.

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	+++

TABLE 22-continued

AAD-1 Event ID Number	Expression Level (ppm)	Western Blot
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)		++++

7.10—Molecular Analysis: Maize Materials and Methods.

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.

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.

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.

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 AfI 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 20×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 1200×100 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

8.1—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 (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 represen-

tative clones of each event with a range of herbicide doses ranging from 1× lethal dose (~⅛× field dose) up to 8× field doses (64× 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.

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 8× field dose (64× 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.

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.

FIG. **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.

Table 23 shows data for the responses of selected AAD-1 (v3)-transformed T_0 corn events to three AOPP herbicides applied postemergence.

TABLE 23

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

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

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

8.2—Field Tolerance of pDAB3404 T_1 Corn Plants to Quizalofop 2,4-D, and Glufosinate Herbicides.

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× 5XH571 is of the same parentage as the AAD-1 (v3) event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

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.

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.

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.

8.2.1—Results.

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.

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.

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.

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).

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 FIG. **16**.

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

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 Evaluation =			Resistance Segregation Ratios following Liberty Spray		
Event or	% 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	AAD-1 Leaf Western	AAD-1 Leaf ELISA	T_1 Population Average		T_2 Population Average
Hybrid	IN	HI	IN	HI	IN	HI	analysis)	Blot T_0	T_0	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 ($P > 0.05$)

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.

8.3—Stacking of AAD-1 (v3) and PAT with Glyphosate Resistance Genes in Corn.

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.

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.

Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. The plants were sprayed with rates of 2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200 mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

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

		% Injury at 14 DAT	
	Field Rate	Hi II X 5XH751 Ave	RR/PAT/AAD1 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

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.

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.

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.

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 (1× field rate, 4× 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 4× lethal dose of quizalofop at the whole plant level.

8.3.3 Expression of AAD-1 (v3) with Respect to Quizalofop Tolerance.

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 FIG. **15**.). The highest tolerance line, event 3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1× 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 1× 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)

9.1—Plant Material.

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.

9.2—Tissue Source.

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.

9.3—Preparation of a Superbinary Vector.

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.

9.4—Bacterial Supply.

All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Pat. No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~1×10^9 cfu/ml) and 100 μM actetosyringone added to the solution.

9.5—Infection and Cocultivation.

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.

9.6—Dose Response Using Immature Embryos.

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 FIG. **17**.

9.7—Selection.

After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the AHAS gene is used, based on U.S. Pat. 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.

9.8.—Regeneration and Seed Production.

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.

9.9—Event Recovery and Analysis Particulars; Whole Plant Screening of T_0 Corn Lineages Containing AAD-1 (v3) and AHAS (pDAS1421).

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 (nM)	Southern Copy # (callus) AAD-1	Western Blot (AAD-1) T_0 Callus	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop Resistant	Sensitive
1421[21]-016	50 Haloxyfop	1	+	8	0
1421[22]-020	100 Haloxyfop	1	++	8	0
1421[22]-022	100 Haloxyfop	1	+	8	0
1421[22]-023	100 Haloxyfop	1	++	8	0
1421[3]-036	100 Haloxyfop	1	++	8	0
1421[4]-031	300 Cyhalofop	1	++	9	0
1421[4]-032	300 Cyhalofop	1	++	8	0
1421[4]-033	300 Cyhalofop	1	++	12	0
1421[4]-034	300 Cyhalofop	1	++	8	0
1421[4]-035	300 Cyhalofop	1	++	6	0

Events with more than 1 copy were not taken to the greenhouse

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

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 (24,000 g) 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 MI/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

Plasmid pDAB3203 containing the AAD-1 (v1) gene was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C. with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C. with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000×g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

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.

Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) 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).

11.1—Extracting AAD-1 (v3) from Plant Leaves.

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 5,000×g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

11.2—Bradford Assay.

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).

11.3—Enzyme Linked Immuno-Sorbent Assay (ELISA).

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).

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

11.4—Western Blotting Analysis.

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

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, Fla.) 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.

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 $\mu E/m^2s^1$).

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.

The DNA samples were diluted to 9 ng/μ1, then denatured by incubation in a thermocycler at 95° C. for 10 minutes.

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

TABLE 27

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

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

nd = not done

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

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.

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.

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.

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 1.5× 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 (~6× 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).

Verification of High 2,4-D Tolerance.

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 187 L/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 200 mM 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 1× field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in

either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by AAD-1 (v3), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

TABLE 28

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

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

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.

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

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.

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).

13.1—Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

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).

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.

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.

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 100×25 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).

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 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.

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.

Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from 10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

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.

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.

13.2—Transformation Method 2: "No-Shake" *Agrobacterium* Mediated Trans-Formation of Non-Regenerable Soybean Suspension Cells.

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.

Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100×25 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.

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 100×15 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 5× concentration of glufosinate ammonium and were maintained for up to 4 months.

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 (FIG. **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%

13.3—Transformation Method 3: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

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

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 ½ B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

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 ½ B5G which contained all ingredients of B5G medium at half strength.

TABLE 30

Growth Media for Soybean

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

Example 14

AAD-1 (v3) Enablement in Cotton

14.1—Cotton Transformation Protocol.

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.

Cotyledon segments (~5 mm) 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.

Agrobacterium Preperation.

Inoculate 35 ml 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.

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.

Larger, well-developed embryos are isolated and transferred to DK media (Table 31) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

TABLE 31

Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1 mM)		1 ml	1 ml	1 ml	4.6 ml		0.5 ml	
2,4-D (1 mM)		1 ml	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 (10 mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

14.2—Experiment Specifics.

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.

14.3—Callus Analysis Results.

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
1	2	pos	***
2	1	pos	***
3	1	pos	***
4	2	pos	***
5	1	pos	***
6	1	pos	neg
7	1	pos	****
8	1	pos	*****
9	2	pos	*
10	1	pos	*

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
11	1	pos	*****
12	1	pos	*
13	1	pos	*
14	1	pos	**
15	1	pos	****
16	1	pos	*****
17	1	pos	*
18	1	pos	***
19	1	pos	**

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
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	**
43	1	pos	*****
44	low DNA	pos	*****
45	1	pos	*****
46	2	pos	*****
47	1	pos	***
48	1	pos	***
49	3	faint pos	neg
50	1	pos	****
51	4	pos	neg
52	2	pos	****
53	1	pos	***
54	1	pos	****
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	*

standard AAD1 5 ug/ml *****
standard AAD1 0.5 ug/ml **

14.4—Plant Regeneration.

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.

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

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.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform AAD-1 (v3), for example, into these and other plants, including but not limited to Maize (*Gramineae Zea mays*), Wheat (*Pooideae Triticum* spp.), Rice (*Gramineae Oryza* spp. and *Zizania* spp.), Barley (*Pooideae Hordeum* spp.), Cotton (*Abroma Dicotyledoneae Abroma augusta*, and *Malvaceae Gossypium* spp.), Soybean (*Soya Leguminosae Glycine max*), Sugar beet (*Chenopodiaceae Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (*Solanaceae Lycopersicon esculentum* and other spp., *Physalis ixocarpa, Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (*Pooideae Secale* spp.), Peppers (*Solanaceae Capsicum annuum, sinense*, and *frutescens*), Lettuce (*Compositae Lactuca sativa, perennis*, and *pulchella*), Cabbage, Celery (*Umbelliferae Apium graveolens*), Eggplant (*Solanaceae Solanum melongena*), *Sorghum* (all *Sorghum* species), Alfalfa (*Leguminosae Medicago sativum*), Carrot (*Umbelliferae Daucus carota sativa*), Beans (*Leguminosae Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (*Leguminosae Pisum, Vigna*, and *Tetragonolobus* spp.), Sunflower (*Compositae Helianthus annuus*), Squash (*Dicotyledoneae Cucurbita* spp.), Cucumber (*Dicotyledoneae genera*), Tobacco (*Solanaceae Nicotiana* spp.), *Arabidopsis* (*Cruciferae Arabidopsis thaliana*), Turfgrass (*Lolium, Agrostis*, and other families), and Clover (*Leguminosae*). Such plants, with AAD-1 (v3) genes, for example, are included in the subject invention.

Example 16

Stacking AAD-1 (v3) with AHAS Herbicide Resistance Gene

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

17.1—AAD-2 (v1) 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 (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.

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at −80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 (200 ng/µl), H_2O 21 Taq polymerase 1 (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.

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.

17.2—Completion of AAD-2 (v1) Binary Vector.

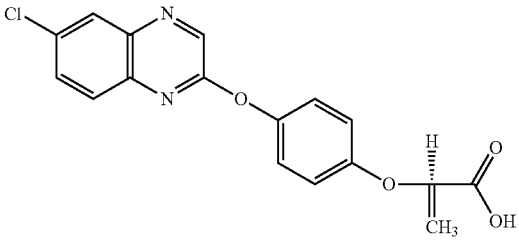
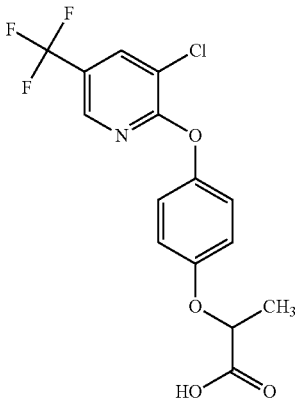
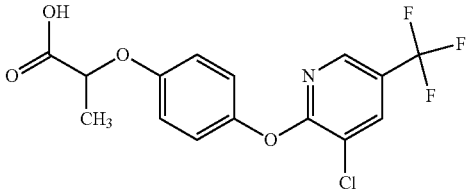
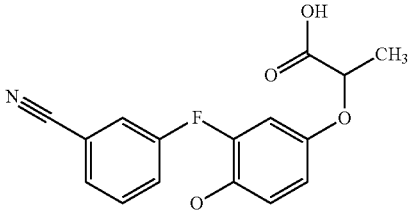
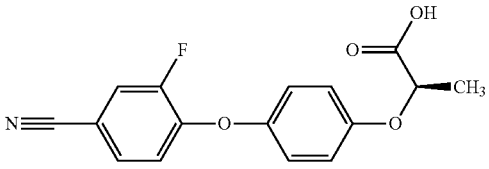
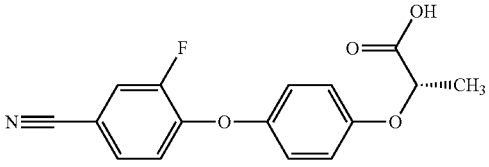
The AAD-2 (v1) gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID 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.*

17.3—Comparison of Substrate Specificities of AAD-2 (v1) and AAD-1 (v1)

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. FIG. **22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D=dichlorprop>(R,S)-haloxyfop>>(R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. Table 33 shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

TABLE 33

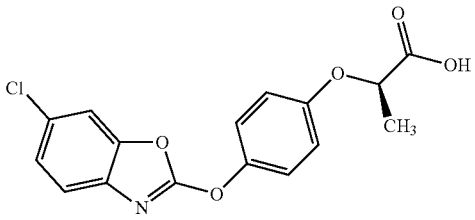
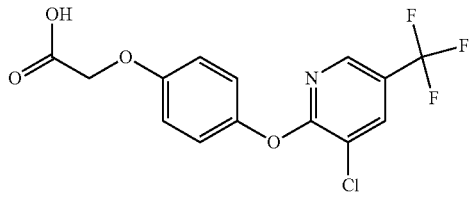
Comparison of AAD1 and AAD2 activity on various substrates.

STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 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

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TABLE 33-continued

Comparison of AAD1 and AAD2 activity on various substrates.

STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
Chiral 	11044492	fenoxaprop	R	0.14	0
	43865	haloxyfop-	—	0	22

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).

The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. See FIG. **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.

17.4—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 (v1) gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), 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.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) 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).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-1f stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C., 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural+supplemental light) at least 1 day prior to testing for the ability of AAD-1 (v3), AAD-1 (v2), or AAD-2 (v1) to provide phenoxy auxin herbicide resistance.

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, Del.) 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).

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).

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.

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 FIG. **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).

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 FIG. **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

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With AAD-1 (v3) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosinate+2,4-D by utilizing products described in federal herbicide

labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed.

Example 19

In-Crop Use of Phenoxy Auxins Herbicides in AAD-1 (v3) Only Transformed Soybeans, Cotton, and Other Dicot Crops

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).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

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.

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

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-1 (v3) would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. AAD-1 (v3)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, et al. could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of AAD-1 (v3)-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3) transformed monocot crop (e.g., corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one

skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

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

The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-1 (v3) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a GT trait are stacked in any monocot or dicot crop species:

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

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AAD-1 (v3)+GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

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

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).

By stacking AAD-1 (v3) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.
   i) Inherently tolerant grass weed species (e.g., *Echinochloa* spp or *Sorghum* spp) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
   ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

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

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like AHAS et al. Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking AAD-1 (v3) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.
   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
   ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
   iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
   iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

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

24.1—Media Description.

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 100×20 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).

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 (PRHSO) 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 (½MSH50).

24.2—Tissue Culture Development.

Mature desiccated seeds of *Oryzu 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 PRHSO pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week prior to storage at 4° C.

24.3—Microprojectile Bombardment.

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).

24.4—Tolerance Testing.

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

24.5—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 scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

24.6—Southern Blot Analysis.

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×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. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Minis TAKRR001A). Primers for Southern fragment PCR AAD-1 were (Forward—ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:31) and (Reverse—GGGCAGGCCTAACTC-CACCAA) (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.

The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in Table 36.

TABLE 36

Southern Results.

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049 bp
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

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.

24.7—Western Data

Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in Table 37.

TABLE 37

Lane	Sample Name	TSP (μg/mL)	ELISA [AAD1] (ng/mL)	ELISA Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	–
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	–
7	AAD1 Standard (0.5 μg/mL)				+++
8	AAD1 Standard (5.0 μg/mL)				+++++

Example 25

Turf Grass Transformation Procedures

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).

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.

25.1—Seed Preparation for Embryogenic Callus Induction.

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).

25.2—Embryogenic Callus Induction.

The culture plates containing prepared seed explants were kept in the dark at room temperature for 6 weeks. Embryo-

genic calli were visually selected and subcultured on fresh callus-induction medium in the dark at room temperature for 1 week before co-cultivation.

25.3—*Agrobacterium* Infection and Co-Cultivation.

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.

25.4—Resting Stage and *Agrobacterium* Control.

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.

25.5—Selection and Potential Transgenic Colony Identification.

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.

25.6—Regeneration of Transgenic Plants.

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).

25.7—Root Induction and Transfer to Greenhouse.

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.

25.8—Vernalization and Out-Crossing of Transgenic Plants.

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.

25.9—Other Target Grasses.

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

26.1—Canola Transformation.

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.

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.

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.

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

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).

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.

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.

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.

26.2—"Molecular Analysis": Canola Materials and Methods

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

26.2.2—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 (Minis 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.

26.3—ELISA.

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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<222> LOCATION: (894)..(919)
<223> OTHER INFORMATION: primer linker

<400> SEQUENCE: 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> SEQ ID NO 6
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: rdpA(ncoI) 5' primer

<400> SEQUENCE: 6

cccatggctg ctgcactgtc ccccctctcc 30

<210> SEQ ID NO 7
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 3'saci primer

<400> SEQUENCE: 7

gagctcacta gcgcgccggg cgcacgccac cga 33

<210> SEQ ID NO 8
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BstEII/ Del NotI 5' primer

<400> SEQUENCE: 8

tggtggtgac ccatccgggc agcggctgca agggcc 36

<210> SEQ ID NO 9
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Sphingobium herbicidovorans

<400> SEQUENCE: 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

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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> SEQ ID NO 10
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v2 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Different from v1
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (212)..(212)
<223> OTHER INFORMATION: Different from v1

<400> SEQUENCE: 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

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> SEQ ID NO 11
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v3 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Different from V1

<400> SEQUENCE: 11

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

<210> SEQ ID NO 12
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 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> SEQ ID NO 13
<211> LENGTH: 289
<212> TYPE: PRT
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 13

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Val

<210> SEQ ID NO 14
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: brjap 5'(speI)

<400> SEQUENCE: 14

actagtaaca aagaaggaga tataccatga cgat 34

<210> SEQ ID NO 15
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: br jap 3' (xhoI)

<400> SEQUENCE: 15

ttctcgagct atcactccgc cgcctgctgc tgc 33

<210> SEQ ID NO 16

<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 forward primer

<400> SEQUENCE: 16

gtaaaacgac ggccagt 17

<210> SEQ ID NO 17
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 reverse primer

<400> SEQUENCE: 17

caggaaacag ctatgac 17

<210> SEQ ID NO 18
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NcoI of Brady

<400> SEQUENCE: 18

tataccacat gtcgatcgcc atccggcagc tt 32

<210> SEQ ID NO 19
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SacI of Brady

<400> SEQUENCE: 19

gagctcctat cactccgccg cctgctgctg cac 33

<210> SEQ ID NO 20
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Glycine max

<400> SEQUENCE: 20

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

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

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

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

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

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

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

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

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

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    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> SEQ ID NO 21
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
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<220> FEATURE:
<223> OTHER INFORMATION: Doubly mutated Soybean EPSPS protein: threonine 183 converted to isoleucine; proline 187 converted to serine

<400> SEQUENCE: 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

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
```

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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> SEQ ID NO 22
<211> LENGTH: 1604
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Soybean-biased DNA sequence encoding doubly-mutated EPSPS disclosed in SEQ ID NO:21, with added sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1575)
<223> OTHER INFORMATION: Coding sequence of soybean-biased DNA sequence encoding the EPSPS protien of SEQ ID NO:21
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1576)..(1578)
<223> OTHER INFORMATION: Translation terminator tga
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1579)..(1604)
<223> OTHER INFORMATION: Sequence included to introduce translation stop codons in all six open reading frames, and to introduce a SacI restriction enzyme recognition site for cloning purposes

<400> SEQUENCE: 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

aggttgacaa agcactgagt agttagctta atcacctaga gctc 1604

<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 5-3

<400> SEQUENCE: 23

agataccctt ggttggttgc 20

<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 3-3

<400> SEQUENCE: 24

cagatggatc gtttggaagg 20

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Lys Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn
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Cys Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe
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Ser Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu
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Phe Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys
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Gln Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val
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The invention claimed is:

1. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R, \text{ wherein}$$

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids.

2. The plant cell of claim **1** wherein said AAD-1 motif has the general formula of:

$$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R(X)_{282\text{-}284}R,$$

wherein $(X)_{282\text{-}284}$ represents a sequence of 3 amino acids.

3. The plant cell of claim **1** wherein the AAD-1 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 9.

4. The plant cell of claim **1** 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.

5. The plant cell of claim **1** wherein said plant cell is a monocotyledonous cell selected from the group consisting of a rice cell and a maize cell.

6. A transgenic plant comprising a plurality of the plant cells of claim **1**, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

7. The plant of claim **6** wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

8. The plant of claim **6** wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

9. The plant of claim **6** wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

10. The plant of claim **6** wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

11. The plant of claim **6** wherein said plant further comprises a second herbicide resistance gene.

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

13. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim **6**, 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 (R)-aryloxyphenoxypropionate herbicide.

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

15. The method of claim **13** wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

16. The method of claim **13** wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

17. The method of claim **13** wherein said method comprises applying a second herbicide.

18. The method of claim **17** wherein said first herbicide and said second herbicide are applied sequentially.

19. The method of claim **17** wherein said first herbicide and said second herbicide are applied concurrently.

20. The method of claim **17** wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

21. The method of claim **17** wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

22. The method of claim **17**, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

23. The method of claim **17**, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

24. The method of claim **17** wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

25. The method of claim **24** wherein said second herbicide resistance gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

26. The method of claim **25** wherein:

(a) said modified ALS gene is selected from the group consisting of SurA, SurB, Csr1, Csr1-1, and Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), GOX, and GAT; and,

(c) said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

27. The method of claim **17** wherein said method further comprises applying a third herbicide.

28. The method of claim **27**, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

29. The method of claim **28** wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

30. A seed comprising a plurality of the plant cells of claim **1**.

31. A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim **30** in said field within 14 days of applying said aryloxyalkanoate herbicide.

32. A plant grown from the seed of claim **30**.

33. A part, progeny, or asexual propagate of the plant of claim **32**, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

34. An (R)-aryloxyphenoxypropionate herbicide tolerant transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzy-

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matically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids, wherein said motif has 90% sequence identity with corresponding amino acids of position 111 to 281 of SEQ ID NO: 9.

* * * * *

EXHIBIT B

Research Article

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Development of enzymes for robust aryloxyphenoxypropionate and synthetic auxin herbicide tolerance traits in maize and soybean crops

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Abstract

BACKGROUND: Effective management of weedy species in agricultural fields is essential for maintaining favorable growing conditions and crop yields. The introduction of genetically modified crops containing herbicide tolerance traits has been a successful additional tool available to farmers to better control weeds. However, weed resistance challenges present a need for additional herbicide tolerance trait options.

RESULTS: To help meet this challenge, a new trait that provides tolerance to an aryloxyphenoxypropionate (FOP) herbicide and members of the synthetic auxin herbicide family, such as 2,4-dichlorophenoxyacetic acid (2,4-D), was developed. Development of this herbicide tolerance trait employed an enzyme engineered with robust and specific enzymatic activity for these two herbicide families. This engineering effort utilized a microbial-sourced dioxygenase scaffold to generate variants with improved enzymatic parameters. Additional optimization to enhance in-plant stability of the enzyme enabled an efficacious trait that can withstand the higher temperature conditions often found in field environments.

CONCLUSION: Optimized herbicide tolerance enzyme variants with enhanced enzymatic and temperature stability parameters enabled robust herbicide tolerance for two herbicide families in transgenic maize and soybeans. This herbicide tolerance trait for FOP and synthetic auxin herbicides such as 2,4-D could be useful in weed management systems, providing additional tools for farmers to control weeds.
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Supporting information may be found in the online version of this article.

Keywords: agriculture biotechnology; herbicide tolerance trait; FOP herbicide tolerance; 2,4-D herbicide tolerance; applied enzyme engineering

1 INTRODUCTION

In agricultural systems, the management of weedy species in crop fields is one of many essential components to ensure favorable crop yields. Many weedy species are well adapted to compete with crops for sunlight, water, soil nutrients, and physical space. Farmers control weeds by utilizing a variety of weed management strategies, including herbicides and genetically modified herbicide-tolerant crops. However, weeds have evolved resistance to herbicides and this continues to present a challenge in agriculture today.[1] One of many strategies to manage resistant weeds is to employ multiple herbicide modes of action.

Two herbicide families of interest are the aryloxyphenoxypropionate (FOP) herbicides and members of the synthetic auxin herbicide family. FOP herbicides inhibit an enzyme variant of acetyl CoA carboxylase (ACCase) found in grasses.[2] Due to this selective nature, FOP herbicides are commonly used to control

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grass weeds in dicot crops. The addition of a FOP herbicide tolerance trait in maize would enable the use of FOP herbicides to control grass weeds in maize fields. The synthetic auxin family of herbicides includes 2,4-dichlorophenoxyacetic acid (2,4-D), which is often used to control dicot weeds.[3] Synthetic auxin herbicides disrupt plant hormone regulatory pathways, resulting in plant death.

Two approaches have been useful in building herbicide-tolerant crops.[4] The first is to utilize a transgene expressing an insensitive variant of an enzyme that is the herbicide's target in a sensitive crop. The second approach is to utilize a transgene expressing an enzyme that deactivates the herbicide chemistry. The oxygenase enzyme α-ketoglutarate-dependent (*R*)-dichlorprop dioxygenase (RdpA), which was isolated from the common soil microbe *Sphingobium herbicidovorans*, could be utilized for the second approach.[5,6] RdpA is part of a large superfamily of dioxygenases found in microbes, plants, and animals.[7,8] RdpA catalyzes the oxidative metabolism of chemistries in the FOP (selective for the *R* enantiomers, the active form of these herbicide chemistries) and synthetic auxin herbicide groups, and has been shown to provide tolerance to these herbicides when expressed as a transgene in sensitive plants.[5,6,9]

A FOP and 2,4-D herbicide tolerance trait, when paired with other herbicide tolerance traits and selective herbicides, can aid in managing and preventing herbicide-resistant dicot and grass weeds, and provides an additional tool for effective weed management. Crops display differences in sensitivity to herbicides in the FOP and 2,4-D families. For example, maize is sensitive to FOP herbicides but displays incomplete tolerance to 2,4-D. Dicots, such as soybeans, are fully tolerant to FOP herbicides, but sensitive to 2,4-D. Therefore, we were interested in engineering an enzyme that could robustly inactivate both FOP and 2,4-D herbicides.

Here, we characterize novel optimized dioxygenase enzyme variants in maize and soybean. These new variants, named FT enzymes (FOP and 2,4-D tolerance enzymes), were engineered to improve enzymatic parameters, including enhanced enzymatic activity on selected herbicides and improved temperature stability of the enzymes. These enhanced variants could be useful in building a new generation of transgenic maize and soybeans with improved herbicide tolerance and agronomic performance when FOP and 2,4-D herbicides are used in a weed control system.

2 MATERIALS AND METHODS

2.1 Purification, enzyme assays and characterization

FT variants were synthesized and cloned into a modified pET28a vector which included a TVMV cleavable N-terminal His tag or a C-terminal His tag (Novagen, Burlington, MA). These variants were transformed into Rosetta 2 cells (Novagen) or BL21 DE3 cells (New England Biolabs, Ipswich, MA) for protein expression overnight at 20 °C. Cell pellets were lysed and FT proteins extracted using B-PER™ and Y-PER™ (ThermoFisher, Waltham, MA) plus lysozyme and benzonase (Sigma Aldrich, St. Louis, MO). Clarified extracts were Immobilized metal affinity chromatography (IMAC) purified using nickel resin (Sigma Aldrich) and eluted using 30 mM Tris pH 8, 200 mM imidazole buffer. Further purification, if needed, included dialysis or size exclusion using 30 mM Tris pH 8 and 200 mM NaCl buffer (ThermoFisher).

Enzyme assays were completed with FT proteins using a modified phenolic-based colorimetric detection assay.[10] Purified proteins (or in the case of the high-throughput screening assays, clarified *E. coli* extracts) were tested with selected substrates. Chemicals were obtained from Sigma Aldrich. For quizalofop (QFOP) and synthetic auxins, *in vitro* assays used the acid form (the active QFOP molecule in plant cells) in the racemic form (if applicable). The reaction buffer contained 20 mM MOPS pH 6.75, 50 µM $(NH_4)_2$ $Fe(SO_4)_2$, 50 µM ascorbic acid (Na salt), and 1 mM α-ketoglutarate (α-KG). Reactions were performed in 150 µL final reaction volumes in 96-well assay plates. All reactions for kinetic characterization were completed with four replications while high-throughput screening reactions for enzyme optimization utilized fewer replications. Reactions were stopped with 15 µL of stop solution (50 mM boric acid, 50 mM KCl, pH 10.0 (KOH) with 2% 4-aminoantipyrine w/v (in water) freshly diluted 1:10 into stop buffer). Color development agent was added (15 µL of an 8% potassium ferricyanide w/v (in water), freshly diluted 1:10). Following color development, assays were recorded with a SpectraMax spectrometer at absorbance of 510 nm. Back calculations were completed with product standards (Sigma Aldrich or custom synthesis) in simulated reactions. Control reactions without α-KG were inactive, and reactions with purified enzyme without $(NH_4)_2$ $Fe(SO_4)_2$ and ascorbic acid were significantly inhibited. Unless indicated, all reactions were completed at 22–23 °C. For the temperature stability assays shown in Fig. 2(b), the enzymes were equilibrated at the indicated temperature for 5 min before substrate (also equilibrated at the same temperature) was added and the reaction allowed to proceed at the same temperature. During the high-throughput screens focused on enhanced temperature stability, screens were run at 25 °C and 40 °C to select variants with strong activity at both temperatures. Kinetic parameters were calculated with nonlinear regression analysis using GraphPad software with default settings.

Protein melting temperatures were determined by fluorescence tracking of the hydrophobic protein binding dye SYPRO Orange (Invitrogen, Waltham, MA) with progressive increases in temperature. The assays were run in a 20 mM Tris, pH 7.75 and 150 mM NaCl buffer. Assays were also run with the addition of $(NH_4)_2$ $Fe(SO_4)_2$ and α-KG since some substrate stabilization was observed. The FT protein and a 1:100 dilution of SYPRO Orange was combined in buffer in a Bio-Rad CFX96 PCR thermocycler with a fluorescence detector. The temperature was progressively increased from room temperature to full protein denaturation. The temperature at which the protein was 50% denatured was calculated from the first derivative of the change in fluorescence in Bio-Rad CFX Manager 2.1 software.

2.2 In-plant controlled environment herbicide tolerance and temperature stability assays in maize

Several different expression vectors containing FT variants were used to transform maize immature embryos using *Agrobacterium*-mediated transformation.[11] Plants containing a single copy of the transgenic insert were identified through molecular and segregation analysis and advanced to seed production for further experimentation.[12] The FT enzymes were expressed either without a targeting peptide or targeted to the chloroplast using the malate dehydrogenase (MDH) chloroplast targeting peptide (CTP).[13] A growth chamber experiment at two different temperatures was conducted to evaluate the tolerance of F1 hybrid transgenic maize expressing MDH/FT_5, MDH/FT_T, MDH/FT_R, FT_T, or FT_R and control plants to postemergence applications of 2,4-D. For QFOP (Assure II®, formulated as an ester in the *R* enantiomer) postemergence tolerance, plants expressing MDH/FT_5, MDH/FT_C, MDH/FT_T, MDH/FT_R, FT_T, or FT_R, and control plants were assayed. The first growth chamber was set to 20 °C

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day and 20 °C night and the second growth chamber was set to 38 °C day and 30 °C night, both with 50% relative humidity and 16-h photoperiod. For each variant/targeting peptide combination tested, three different transformation events were evaluated with eight replications for each temperature regime. The herbicide treatment consisted of 2,4-D amine at 4500 g ha^{-1} (4X) applied to V3/V4 maize and QFOP at 360 g ha^{-1} (4X) plus 0.25% v/v Chemsurf™ nonionic surfactant (NIS) containing a 90% mixture of alkylarylpolyoxykane ether, isopropanol and free fatty acids applied to V2 maize in a track sprayer. Plants were visually rated 11 days after treatment (2,4-D) and 9 days after treatment (QFOP) on a scale of 0% to 100% with 0% being no injury and 100% being plant death. This scoring system is based on classical herbicide injury rating systems and includes injury to plant tissue such as chlorosis, necrosis, malformation, stunting and plant death. Injury data were averaged with standard error bars calculated in Excel.

2.3 Field-based herbicide tolerance assays in maize

Field experiments were conducted at two independent locations in Illinois, USA, to evaluate herbicide tolerance of maize expressing MDH/FT_5, MDH/FT_C, MDH/FT_T, MDH/FT_R, FT_T, or FT_R, and control maize to postemergence applications of QFOP and 2,4-D. Experimental design was a randomized complete block design with two replications. For QFOP, herbicide treatments consisted of (i) QFOP at 360 g ha^{-1} plus 0.25% v/v NIS applied to maize multiple times within the same plot, VE-V2 followed by (fb) V4 fb V8, (ii) QFOP at 720 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize, and (iii) QFOP at 1440 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize. For 2,4-D, herbicide treatments consisted of (i) 2,4-D amine at 2250 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 followed by (fb) V4 fb V8 maize, (ii) 2,4-D amine at 4500 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize, (iii) 2,4-D amine at 9000 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize, and (iv) 2,4-D amine at 18 000 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize. Herbicide treatments were applied with a CO_2 backpack or tractor-mounted sprayer calibrated to deliver 140 L ha^{-1} using air-inducted Teejet® TTI nozzles with water as the herbicide carrier. Plots were visually rated 10–14 days after V4 and V8 applications for crop injury. All data were subjected to analysis of variance and means separated at LSD or P value < 0.05.

2.4 In-plant herbicide tolerance in soybeans

Several different vectors containing FT variants were transformed into soybean using *Agrobacterium*-mediated transformation. Individuals (a transformation event) containing a single copy of the transgenes were identified and plants were advanced to seed production for further experimentation. Homozygous FT expressing plants were grown to the V3 growth stage and treated with 1680 g ha^{-1} of 2,4-D amine in controlled environment studies. Plants were assessed for visual injury approximately 7 days after treatment. Non-traited control is an average injury of four plants and the experimental treatments are the average injury of 19–39 plants. In field treatments, homozygous FT expressing plants were treated with 2240 g ha^{-1} of 2,4-D at V3 and R1 growth stages.

2.5 Data availability

Sequence information is available in GenBank:

FT_5: MH043108; FT_C: MH043109; FT_R: MH043110 and MH043111; FT_T: MH043112 and MH043113; FT_Tv3: MH043114; FT_Tv7: MH043115. Records for RdpA (AAM90965) and MDH (BT000621) have been previously submitted by other groups.[5,13]

The crystallography data referred to in this study are available in the Protein Data Bank (PDB, https://www.rcsb.org/):

6D0O, RdpA dioxygenase holoenzyme; 6D1O, FT_5 dioxygenase apoenzyme; 6D3H, FT_T dioxygenase with bound dichlorprop; 6D3I, FT_Tv7 dioxygenase with 2,4-D bound; 6D3J, FT_T dioxygenase holoenzyme; and 6D3M, FT_T dioxygenase with bound QFOP.

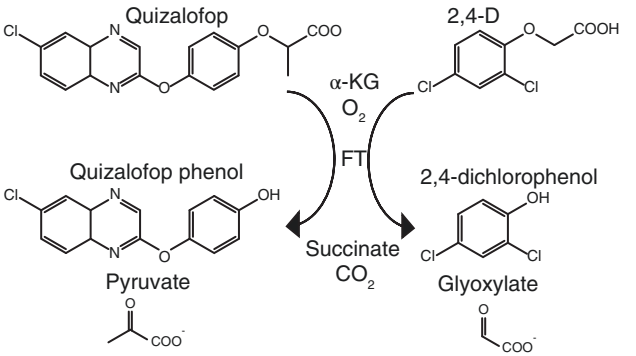


Figure 1. FT enzymes catalyze herbicide deactivation. The dioxygenase, FT, catalyzes the initial metabolism of chemistries in two herbicide chemistry families: FOP, represented by quizalofop (QFOP), and members of the synthetic auxins, represented by 2,4-D.

3 RESULTS

3.1 Introduction

The dioxygenase RdpA has been characterized as an α-ketoglutarate-dependent non-heme iron-dependent dioxygenase.[6] This enzyme utilizes α-KG, molecular oxygen and a variety of herbicide compounds to catalyze a classical dioxygenase reaction (Fig. 1). This reaction results in the release of CO_2, succinate and two breakdown products from the herbicide chemistry. However, the natural enzymatic activity of RdpA could be improved through enzyme engineering to provide tolerance to both herbicide chemistries in both monocot and dicot crops in typical field conditions. An enzyme engineering effort was thus undertaken to design optimized enzymes with enhanced activity on selected herbicide chemistries. RdpA was used as a base dioxygenase scaffold for engineering design and the optimized FT enzyme variants were screened for robust enzymatic activity on the herbicides QFOP, a FOP herbicide, and 2,4-D, a synthetic auxin. In-plant analysis of maize and soybean plants expressing selected FT variants in both controlled environment and field-based testing was essential for informing additional optimization parameters. This included a need to stabilize the enzyme for field temperature extremes and optimizing the activity of the enzyme towards the substrate 2,4-D.

3.2 Engineered FT variants demonstrated FOP tolerance in maize, but low enzyme temperature stability

At the outset of this engineering effort a series of now well-established engineering approaches were applied to RdpA to optimize activity for QFOP and 2,4-D separately and in combination (see the supporting inofrmation). Engineering approaches included homology modeling and homologous amino acid changes, scanning mutagenesis, computational protein design, rational protein design and data-driven design. At the onset of the engineering, no crystal structure of RdpA or a close

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homolog was available and therefore a homology model was generated with ICM-pro using a distant dioxygenase homolog, TauD (30% sequence identity), as a template.[14] This homology model was used for substrate docking and identification of putative active site residues for rational design to modulate protein–substrate interactions. As discussed below, we later used structural characterization of enzyme variants as crystal structures were determined to help inform additional engineering. This coupled with the enzymatic characterization of early variants described below helped further drive the computational, rational and data-driven design efforts of additional optimized variants.

The enzyme variants were assayed in a bacterial expression system and tested in a high-throughput, plate-based enzymatic assay using clarified bacterial lysates. This assay enabled screening of approximately 4000 enzyme variants in a semi-quantitative manner. Those variants identified with improved activity relative to bacterial lysate expressing RdpA were further combined in multiple rounds of optimization. The highest performing variants in the bacterial lysate screen (depending on the optimization objectives described below) were confirmed with purified protein assays. A small subset (<1% in total) of these confirmed variants were selected for *in planta* efficacy analysis.

The enzyme variant FT_5 was the first variant selected for in-plant analysis based on first round *in vitro* kinetic characterization. The FT_5 variant showed good activity with QFOP (0.44 μmol mg^{-1} min^{-1} at V_{max}) and weaker activity with 2,4-D (0.03 μmol mg^{-1} min^{-1} at V_{max}) in enzyme assays. Although this activity was lower than that observed for RdpA, FT_5 was selected early in the optimization process to benchmark in-plant tolerance assays and to direct the optimization efforts. A transformation vector with the FT_5 transgene linked to a plant CTP at the N-terminal end of the enzyme was used to transform maize. A CTP was included because FOP herbicides inhibit the enzyme ACCase. In grasses such as maize, the herbicide sensitive ACCase enzyme isoform is localized to the chloroplast.[15] Therefore, we hypothesized that localizing the FT_5 enzyme to the chloroplast would provide improved FOP tolerance.

Maize immature embryos from the germplasm LH244 were transformed with FT_5 transformation vectors using *Agrobacterium tumefaciens*. The regenerated transformed plants were spray challenged with a commercial formulation of QFOP. Following spray applications, the transformed plants were rated for tolerance using a percentage injury rating. FT_5 plants showed tolerance to the herbicide application while the control plants showed extensive tissue damage and eventual plant death (Fig. 2(a)). Treated plants with low injury ratings were grown to set seed and advanced for additional testing in the following generations.

Greenhouse testing for FT_5 was encouraging; however, a robust herbicide tolerance trait requires good performance in field-relevant environmental conditions. Therefore, the FT_5 maize plants were screened for herbicide tolerance in Midwestern USA field research plots that mimicked typical maize cropping conditions. In these trials, FT_5 expressing plants were tolerant to both QFOP and 2,4-D, showing that the FT proteins can provide tolerance to these herbicides in field conditions (Fig. 3(a) and (c)). However, tolerance in field conditions using the FT_5 variant was not as robust as desired, with injury rates exceeding 20% in some treatments. Therefore, additional characterization of the FT_5 enzyme variant and FT_5 maize plants was undertaken.

Further FT_5 enzyme characterization showed that FT_5 was unstable at elevated temperatures *in vitro* and in plants,

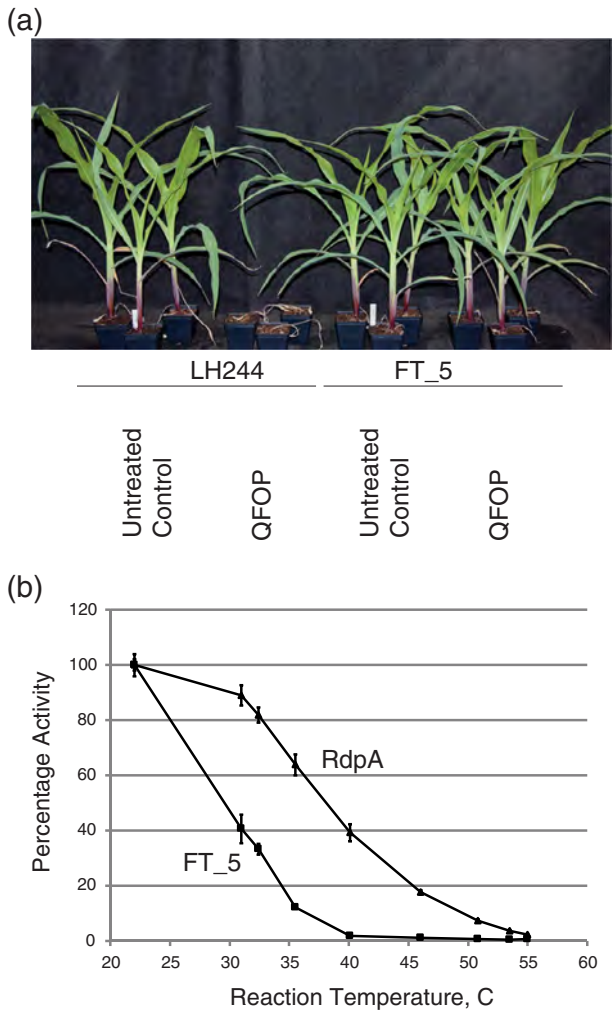


Figure 2. Characterization of FT_5. (a) Plants expressing FT_5 showed tolerance to QFOP applications in controlled environment experiments. Plants were treated with QFOP at 90 g ha^{-1}. The transformation background, LH244, served as a sensitive control while LH244 plants expressing the FT_5 enzyme (FT_5) were the test plants. Image taken 18 days after treatment. (b) Enzyme activity of FT_5 relative to RdpA with QFOP. Both FT_5 and RdpA show a progressive loss of activity with increasing reaction temperature (relative to room temperature control, which was set at 100% for both enzymes). Error bars indicate the standard error (SEM) of three replications.

suggesting that temperature stability is essential for improved in-crop performance. In the field environment, plants are exposed to large temperature swings from night to day and throughout the season. In addition, daytime temperatures in Midwestern fields commonly exceed 30 °C and can exceed 37.5 °C, the leaf surface temperature at which net photosynthesis begins to decline in well-watered maize plants.[16] A robust herbicide tolerance trait is dependent on a tolerance enzyme that can function throughout a real-world temperature range. FT_5 enzyme characterization revealed a progressive loss of enzyme function at temperatures above 30 °C with nearly complete enzyme inactivity at 40 °C with *in vitro* testing (Fig. 2(b)). While the original RdpA enzyme variant displayed somewhat more temperature stability *in vitro* than FT_5, RdpA was also temperature sensitive, with nearly 40% loss of activity at 35 °C (Fig. 2(b)). We also pre-exposed the FT_5 enzyme to an elevated temperature of 35.5 °C (a temperature which results in nearly 90% loss of activity) for various time lengths of 0 to 35 min before returning the enzyme to 22 °C for

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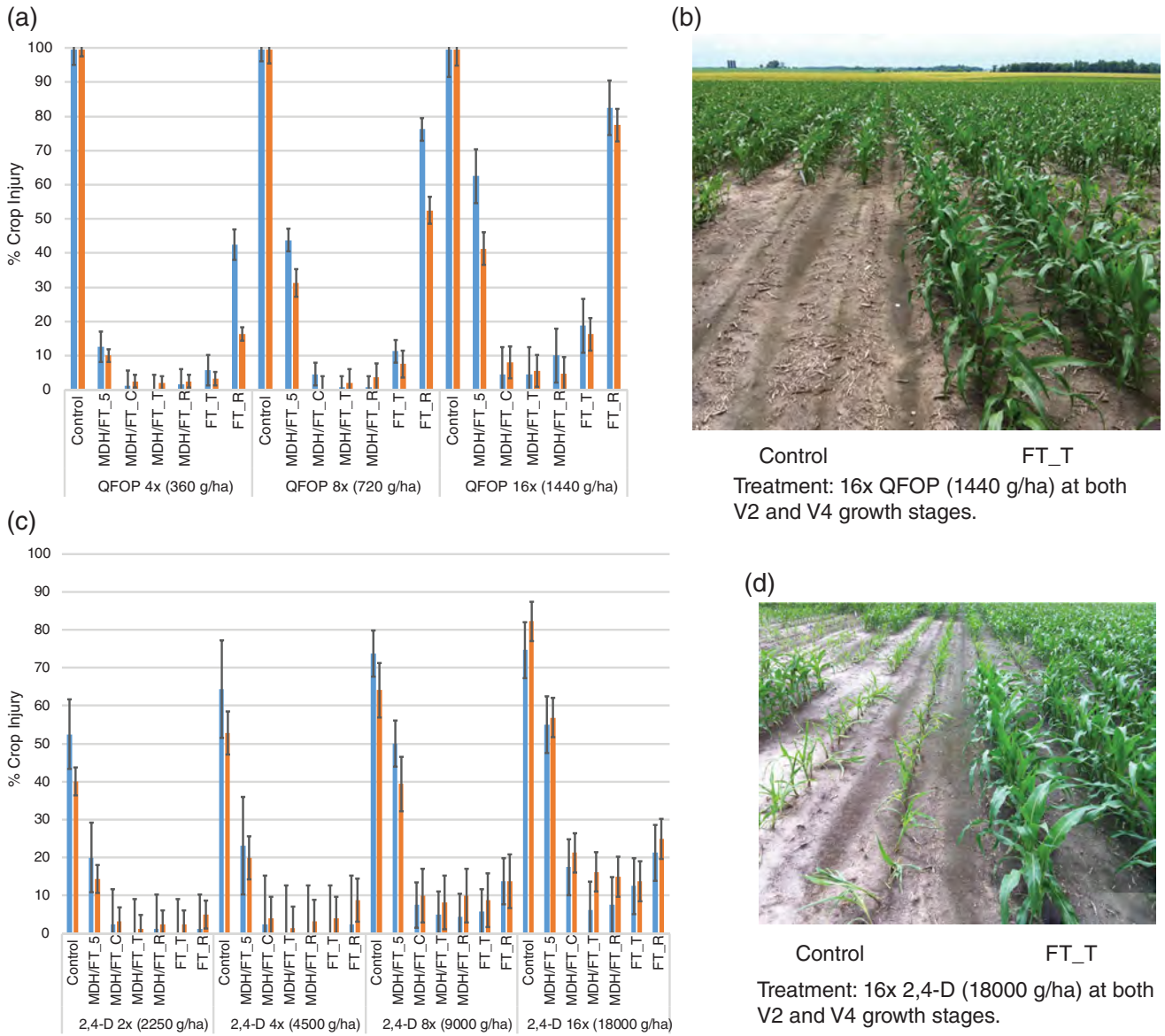


Figure 3. Field testing of FT variants. (a) Field testing confirms robust QFOP tolerance in maize. F1 plants expressing the indicated FT variants were treated at V2 and V4 (early vegetative) and V8 (late vegetative) with the indicated QFOP treatments. Injury scores were taken approximately 1 week post-treatment. The temperature-stable variants performed well when paired with a CTP. Injury ratings increased when the enzymes were expressed without a CTP. The blue bars indicate crop injury following the V4 treatments while the orange bars are following the V8 treatment, error bars indicate LSD at $P < 0.05$ with four replications. (b) Field view of MDH/FT_T (chloroplast targeted FT_T) expressing plants following the V4 application of QFOP. (c) Field testing confirmed robust 2,4-D tolerance. F1 plants expressing the indicated FT variants were treated at V2, V4 and V8 with the indicated 2,4-D treatments. Injury scores were taken approximately 1 week post-treatment. The temperature stabilized variants performed well in this assay either with or without a CTP. The blue bars indicate crop injury following the V4 treatments while the orange bars are following the V8 treatment, error bars indicate LSD at $P < 0.05$ with four replications. (d) Field view of MDH/FT_T expressing plants following V4 2,4-D application.

a traditional activity assay with QFOP. In this assay, the 10-min heat pre-treatment reduced enzyme activity by 50%, suggesting that the loss of activity at elevated temperatures is at least partly irreversible (data not shown).

To confirm that the temperature sensitivity observed in the FT_5 *in vitro* assays correlated with incomplete tolerance in plants, a series of growth chamber treatments was undertaken. Maize seedlings were moved to two temperature regimes before application of QFOP or 2,4-D herbicides. The first was a 20 °C day and night regime (non-stressed) and the second was a 38 °C day and 30 °C night regime (heat stress). FT_5 transgenic plants displayed tolerance to both herbicide applications at the non-stressed temperature regime, but in the heat stress regime these plants demonstrated increased plant injury for both herbicides (Fig. 4). Together, the *in vitro* and in-plant results confirmed that temperature sensitivity of the FT_5 enzyme contributed to incomplete FOP and 2,4-D herbicide tolerance that was observed in maize field experiments. Therefore, enzyme optimization was conducted with a focus on improving temperature stability.

3.3 Crystal structure analysis informed subsequent designs

To enhance our ability to engineer additional FT enzyme variants, crystal structures of several FT enzymes (discussed below) were analyzed. The engineered FT enzyme variants and RdpA were

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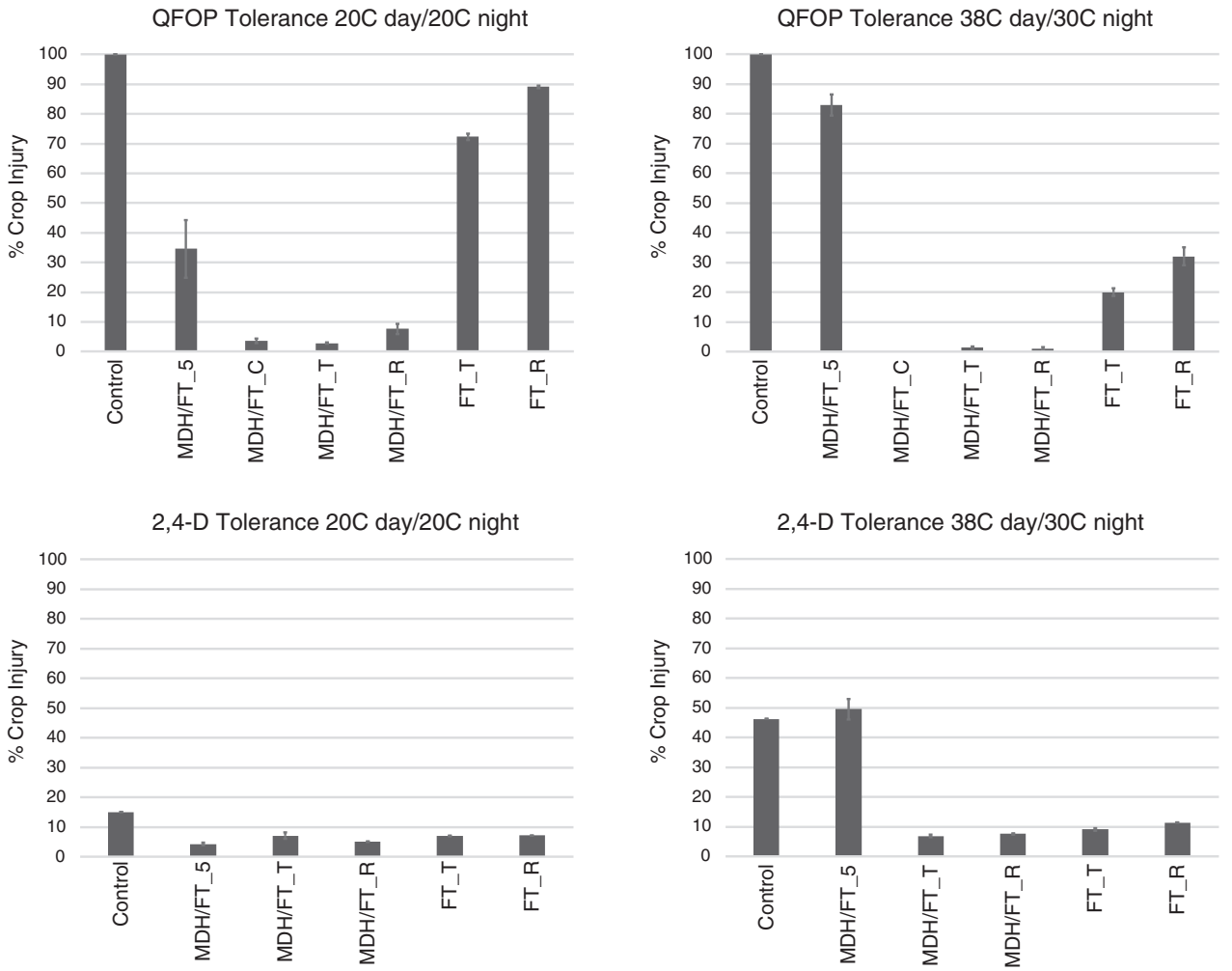


Figure 4. In-plant testing of FT enzymes in controlled environments. Controlled environment assays demonstrated differences in temperature stability of FT enzymes in maize plants. Plants were screened in non-stressed and heat-stressed conditions. Plants were grown to approximately V2 growth stage (early vegetative) and then moved to one of the two temperature regimes. After 2 days of acclimation, the plants were treated with either QFOP (4X, 360 g ha^{-1}) or 2,4-D (4X, 4500 g ha^{-1}) applications and rated for percentage injury 1 week after spray. Mean injury scores for eight replications shown with SEM indicated.

crystalized in a homotetramer asymmetric unit that contained a classical dioxygenase active pocket including a cobalt atom (replacing an iron atom found in the biologically active enzyme) coordinated by two histidine residues and one aspartic acid residue (Fig. 5 and see the supporting information, Fig. S1(a–d)). The substrates QFOP and α-KG were found to dock in the active pocket in an expected orientation for dioxygenase enzymes. A flexible loop caps the active pocket and was only observed to be well-ordered in crystal structure analysis when the enzyme contained a cobalt atom and both α-KG and a herbicide substrate.

Crystal structure analysis of the FT_5 enzyme was attempted to get a better understanding of the temperature instability. However, FT_5 was found to crystalize poorly, likely due to the instability of the enzyme (Fig. S1(e)). The enzyme could not be co-crystalized with the metal atom or substrates, and the flexible loop that was observed to become well-ordered in the crystal structures of other more stable FT variants upon substrate binding remained disordered in the FT_5 crystals. This disordered nature of the FT_5 enzyme is consistent with the inherent instability observed at higher temperatures in the enzyme assays and supports our hypothesis that the instability of FT_5 is a primary cause

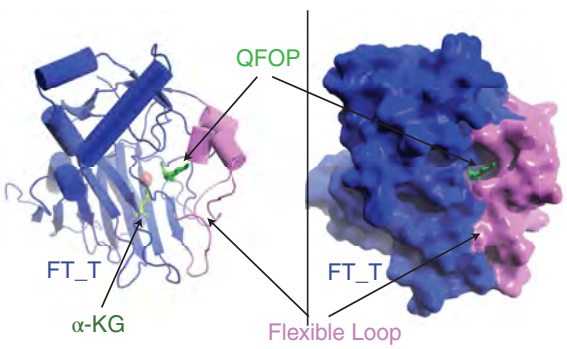


Figure 5. FT enzyme crystal structure. X-ray crystallography was used to solve the structure of FT enzymes (FT_T shown in both ribbons and space fill views). The binding of the co-substrates α-KG and QFOP (shown in light and dark green) resulted in an ordering of loops surrounding the active pocket (highlighted in purple) that also contained a metal ion. Cobalt is used to replace the iron atom during crystallization. The view as a monomer is shown for clarity.

of reduced tolerance at higher temperatures in plants. Comparison of crystal structures of the engineered variants and RdpA helped enable additional engineering.

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Table 1. Characterization of selected FT enzyme variants

			RdpA	FT_R	FT_T	FT_Tv3	FT_Tv7
		Buffer	43	55	58	57	57
	Protein melting	Buffer (Fe and α-KG)	53	58	62	62	61
FOP	Quizalofop	V_{max}	2.76 (0.11)	1.60 (0.05)	1.62 (0.05)	1.51 (0.05)	1.53 (0.05)
		K_m	0.09 (0.01)	0.09 (0.01)	0.12 (0.01)	0.12 (0.01)	0.14 (0.01)
		k_{cat}/K_m	1.04	0.61	0.46	0.43	0.37
Synthetic auxins	Dichlorprop	V_{max}	5.32 (0.09)	3.03 (0.06)	2.09 (0.03)	1.29 (0.02)	1.04 (0.01)
		K_m	0.06 (0.004)	0.06 (0.01)	0.03 (0.002)	0.04 (0.003)	0.03 (0.002)
	2,4-D	V_{max}	0.25 (0.01)	0.63 (0.02)	1.17 (0.01)	3.38 (0.05)	2.91 (0.04)
		K_m	0.13 (0.02)	0.05 (0.01)	0.03 (0.002)	0.08 (0.004)	0.07 (0.004)
		k_{cat}/K_m	0.06	0.43	1.33	1.44	1.42
	MCPA	V_{max}	0.20 (0.01)	ND	1.23 (0.02)	3.64 (0.07)	2.93 (0.06)
	Mecoprop	V_{max}	4.11 (0.15)	ND	3.04 (0.07)	1.78 (0.03)	1.28 (0.02)

Lead FT variants and RdpA as a control are characterized for enzyme temperature stability and enzyme kinetics with FOP and synthetic auxin herbicides. Protein melting is recorded as the temperature (°C) in which the protein is 50% denatured in buffer alone or with supplemental Fe(II) and α-KG. Enzyme kinetic parameters are recorded for representative FOP and synthetic auxin herbicides with V_{max} (μmol mg^{-1} min^{-1}), K_m (mM) and k_{cat}/K_m (M^{-1} min^{-1}) shown. The standard error is shown in parenthesis. ND, not determined.

3.4 Temperature stable FT variants are identified

To engineer variants with improved temperature stability, structure-based design was applied in addition to the engineering approaches described above. Structure-based design leveraged a focus on the loops identified above and computational design. High-throughput screens were modified to include elevated temperatures and data-driven design enabled combinations of positive variants.

The temperature stable FT_C variant was identified with enhanced temperature stability *in vitro*. FT_C had an approximately 20 °C increase in protein melting temperature over FT_5 and maintained enzymatic activity towards QFOP (data not shown). Therefore, the FT_C variant was used as a base variant for enzyme engineering with the goal of maintaining good temperature stability while improving enzyme activity, particularly for 2,4-D as a substrate. We hypothesized that optimizing 2,4-D activity would be essential for building a 2,4-D tolerance trait in soybeans, a crop that is more sensitive to this herbicide relative to maize.

The new engineered FT variants, FT_T and FT_R, showed enhanced temperature stability with improved enzymatic activity (Table 1). Activity assays with QFOP and 2,4-D as substrates were completed with variants FT_T and FT_R (with RdpA as a control) in a range of reaction temperatures. In these assays, RdpA lost >50% activity towards QFOP at 40 °C while both FT_T and FT_R maintained >50% activity at 40 °C relative to control reactions at 25 °C (data not shown). Activity assays were also completed in which the FT_T and FT_R variants were preheated at selected temperatures (25–95 °C) before returning to 25 °C for an activity assay with QFOP. In these assays, both variants maintained approximately 50% activity with a 45 °C preheat (relative to the 25 °C preheat), further demonstrating robust temperature stability over a broad temperature range (Fig. S2). The enzymes were irreversibility inhibited at temperatures above 75 °C in this assay, consistent with the observed protein melting temperatures of approximately 60 °C (Table 1). In addition, enzymatic analysis using HPLC and mass spectrometry to confirm the reaction products demonstrated that FT_T metabolized QFOP and 2,4-D in the same manner as RdpA (Fig. S3).

3.5 FT variants with optimized 2,4-D activity are identified

FT_T showed a distinct improvement in activity towards 2,4-D relative to RdpA in the *in vitro* assays (Table 1). However, we were interested in additional engineering to determine if 2,4-D activity could be further optimized to enhance activity and crop tolerance, especially for soybean. Additional enzyme engineering, using FT_T as a base scaffold with computational protein design focused on substrate binding, resulted in the creation of variants, including FT_Tv3 and FT_Tv7. These variants had enhanced enzymatic activity when tested with 2,4-D as a substrate (Table 1), activity levels similar to SdpA, a dioxygenase characterized as having stronger activity on 2,4-D relative to RdpA (data not shown;[6,9]).

The FT variants were further characterized with FOP and auxin family herbicide chemistries (Table 1). The FT variants displayed lower activity with QFOP relative to RdpA. However, as discussed below, FT variants' FOP activity was suitable for robust in-plant tolerance, but 2,4-D activity needed to be optimized. The optimized FT variants showed enhanced 2,4-D activity with FT_Tv3 and FT_Tv7 showing a greater than 10-fold V_{max} improvement over RdpA. The 2,4-D and MCPA chemistries have two-carbon side chains that are cleaved by FT, while dichlorprop and mecoprop have three-carbon side chains identical to the FOP chemistries. The FT_Tv3 and FT_Tv7 variants have been optimized with a modified substrate preference based on the cleaved side chain, relative to RdpA. During structural analysis, several unsuccessful attempts were made to co-crystalize RdpA or FT_T with 2,4-D. FT_Tv7 was successfully co-crystalized with 2,4-D, suggesting that this variant binds 2,4-D in its active pocket better than RdpA or FT_T (Fig. S1(f)).

3.6 In-plant controlled environment and field testing demonstrates robust performance of FT variants

In-plant testing was used to further characterize selected variants. In maize, the temperature-stable variants displayed improved heat tolerance in controlled growth chamber experiments with low

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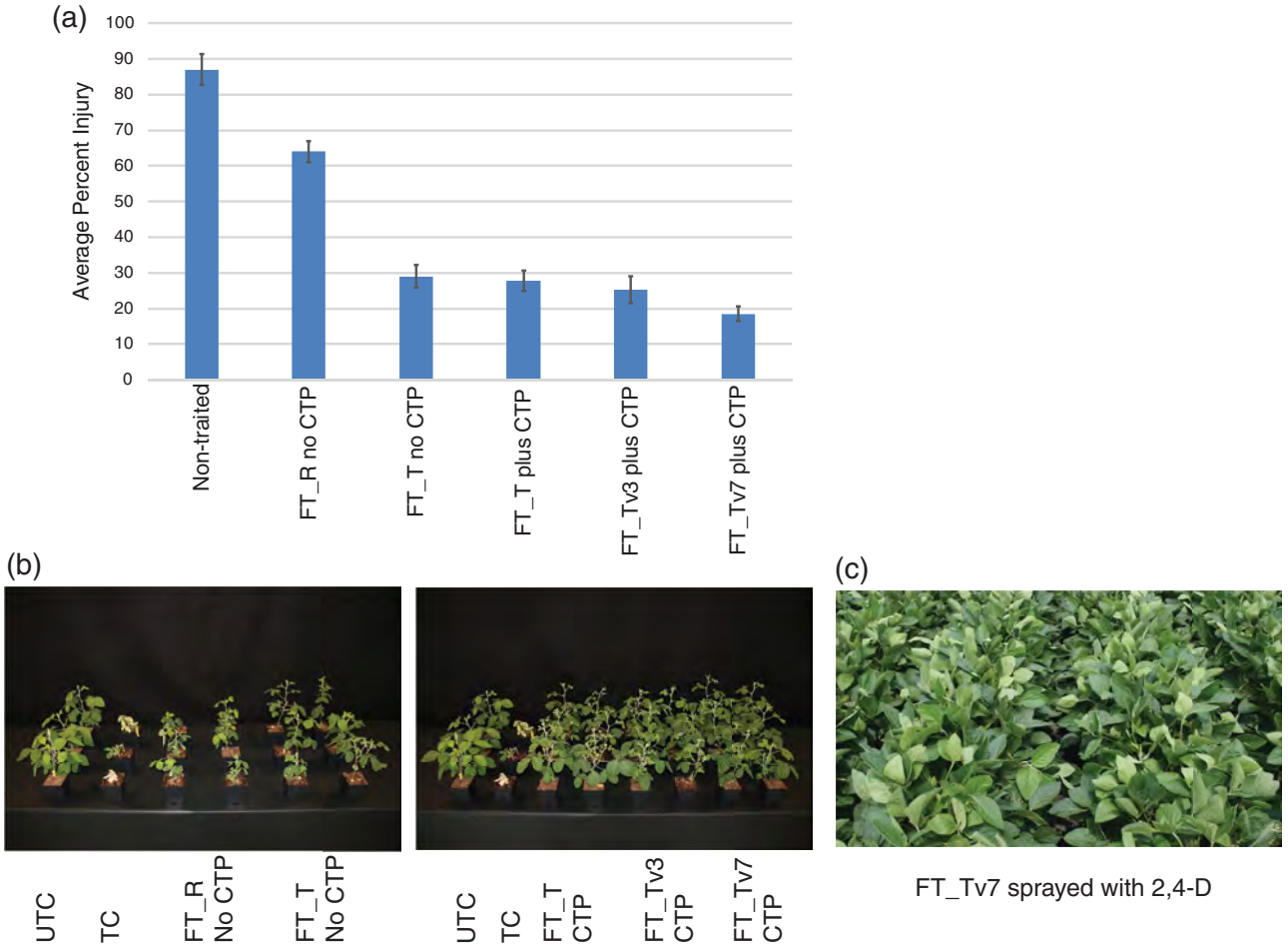


Figure 6. FT enzymes enable robust 2,4-D tolerance in soybeans. (a) Greenhouse 2,4-D spray assays in soybean demonstrated tolerance to 2,4-D in soybeans expressing FT_T, FT_Tv3 or FT_Tv7, with or without a CTP relative to control plants. Soybean lines expressing the indicated FT variant, or control plants, were treated with 2,4-D (1680 g ha^{-1}). Error bars indicate SEM. (b) Images of representative plants from (a). UTC, untreated control plants; TC, treated control plants. Control plants are in single rows of three plants; experimental plants are in double rows of three plants each. Treated control plants show extreme stunting, tissue necrosis and chlorosis. FT_R plants show some stunting and chlorosis. FT_T, FT_Tv3 and FT_Tv7 plants only show limited epinasty/malformation of upper stems and leaves. (c) Field view of FT_Tv7 expressing soybean plants following two applications (V3 and R1 growth stages) of 2,4-D (2240 g ha^{-1}) demonstrating 2,4-D tolerance.

injury ratings for both the non-stressed and heat-stressed treatments (Fig. 4). This observation confirms that the *in vitro* temperature stability of these enzyme variants directly translated to tolerance stability in plants.

Field plots with maize expressing the indicated variants were sprayed with QFOP and 2,4-D herbicides. Both QFOP and 2,4-D were tested at rates up to 16-fold over recommended agricultural field rates with repeated applications at several growth stages. In the treatments with QFOP, the control plants were completely killed while the temperature-stable variants (FT_C, FT_T and FT_R) showed little injury (Fig. 3(a) and (b)). FT_5 showed injury that increased at higher herbicide rates, demonstrating incomplete tolerance. In treatments with 2,4-D, a similar trend as with QFOP was observed (Fig. 3(c) and (d)). Maize is naturally partially tolerant to 2,4-D so complete plant death was not observed, but typical 2,4-D injury, including malformation, lodging and stunting in the control plants, was observed. Plants expressing the temperature-stable variants displayed excellent tolerance to 2,4-D, while plants expressing the FT_5 variant demonstrated incomplete tolerance at high 2,4-D rates. This demonstrated that the temperature-stable variants provided excellent tolerance to both QFOP and 2,4-D herbicide applications under field conditions in maize. Maize plants were also sprayed with clethodim, a herbicide with the same mode of action as QFOP, but a different chemical family (cyclohexanedione, DIM). As expected, no tolerance was observed for clethodim-treated FT_T expressing maize plants (data not shown). Therefore, DIM herbicides can control volunteer maize plants expressing FT in a maize-soybean crop rotation.

Maize plants were transformed to express FT variants coupled with the MDH CTP or without a CTP. As discussed above, the in-plant assays focused on using FT enzymes coupled with a CTP based on the chloroplast localization of the herbicide target. The mode of action for 2,4-D is not known to target the chloroplasts, suggesting a CTP was unlikely to improve 2,4-D tolerance.[17,18] To test these hypotheses on sub-cellular targeting, maize plants were also generated expressing FT enzymes without a CTP. The CTP-targeted FT demonstrated robust tolerance to both QFOP and 2,4-D, while the non-targeted versions of the FT enzymes (no targeting peptide) showed robust 2,4-D tolerance with reduced QFOP tolerance (Fig. 4). In the high-temperature regime, rapid growth rates appeared to somewhat, but not fully, compensate for QFOP injury observed in the non-targeted FT expressing plants. In the field-based testing, greater injury was also observed at higher QFOP rates in plants expressing FT without a CTP than FT

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with a CTP (Fig. 3(a)), further suggesting that proper sub-cellular targeting of the FT enzyme improves tolerance to FOP herbicides. In addition, we also noted some increase in FT enzyme expression when FT is coupled with a CTP, suggesting this may, in part, contribute to enhanced tolerance. However, in the treatments with 2,4-D, FT enzymes with or without a CTP showed comparable performance, suggesting that the 2,4-D chemistry can be effectively deactivated both within and outside the chloroplast to provide 2,4-D herbicide tolerance (Fig. 3(c)).

In maize, the FT variants provided robust tolerance to 2,4-D herbicide applications. However, we were also interested in determining if the improved 2,4-D activity characterized for some of the temperature-stable FT variants could provide tolerance in soybeans to 2,4-D (a more sensitive crop). Therefore, we transformed a commercially relevant soybean line, A3555, using *Agrobacterium*-mediated transformation into excised embryos with several different FT enzyme variants, both with and without a CTP. Soybean plants expressing these variants were treated with 2,4-D applications. Similar to what was observed in maize, the presence or absence of the CTP did not significantly change plant 2,4-D tolerance. FT_R, a variant with improved 2,4-D activity relative to RdpA (but less activity than FT_T, FT_Tv3 and FT_Tv7; Table 1), was unable to provide good 2,4-D tolerance in soybean, with injury rates above 50%. However, FT_T, FT_Tv3 and FT_Tv7 demonstrated 2,4-D tolerance in soybean with injury rates below 30% (Fig. 6(a) and (b)). FT_Tv7 also showed commercially relevant 2,4-D tolerance in field applications of 2,4-D (Fig. 6(c)). These data support the hypothesis that engineering FT variants with optimized 2,4-D activity was essential to providing full tolerance in crops, especially sensitive crops such as soybean.

4 DISCUSSION AND CONCLUSIONS

Successful weed management is essential for ensuring robust crop yields. Here we report the successful development of engineered FT enzymes that can provide herbicide tolerance in crops to two distinct families of herbicides. Weed resistance for many herbicide families (including FOP and synthetic auxins) is already present in agricultural production areas. Therefore, successful utilization of this trait, and any herbicide tolerance trait, requires a systems-based approach to weed management. This approach includes careful consideration of the weeds present, the biology and herbicide resistance of the weed populations, the agronomic and cultural practices available and the use of combinations of herbicides encompassing multiple modes of action. The FT herbicide tolerance trait can be paired with other herbicide tolerance traits and other selective herbicides to enable additional options for farmers in their holistic weed management systems.

Ever-expanding genomics and proteomics knowledge from a variety of sources, including microbes, provides a rich source of enzyme scaffolds that can be utilized in biotechnology to design optimized enzymes. These resources can be leveraged in the optimization of enzymes for agronomically relevant properties, such as temperature stability of the FT enzymes, which may be essential for robust performance in a field setting where plants are exposed to wide temperature ranges. Another optimization target is modulating enzymatic activity with substrates of interest. For example, the FT enzymes provide tolerance to members of two herbicide families, FOP (QFOP) and synthetic auxin (2,4-D) herbicides. In conclusion, a microbial enzyme scaffold served as a starting point for the engineering of FT enzyme variants that were optimized for both enzyme activity on preferred herbicide chemistries and for enzyme temperature stability. In-plant analysis demonstrated that both elements were essential to provide robust herbicide tolerance to two herbicide families in maize and soybean crops.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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EXHIBIT C

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Here's how you know

Synthetic construct FT_T_m gene, complete cds

GenBank: MH043112.1

FASTA Graphics

Go to:

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LOCUS       MH043112                888 bp    DNA     linear   SYN 28-APR-2018
DEFINITION  Synthetic construct FT_T_m gene, complete cds.
ACCESSION   MH043112
VERSION     MH043112.1
KEYWORDS    .
SOURCE      synthetic construct
  ORGANISM  synthetic construct
            other sequences; artificial sequences.
REFERENCE   1  (bases 1 to 888)
  AUTHORS   Larue,C.T.
  TITLE     Development of Robust FOP and Synthetic Auxin Herbicide Tolerance
            Traits for GE Crops
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 888)
  AUTHORS   Larue,C.T.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-MAR-2018) CV, Monsanto Company, 700 Chesterfield PKWY
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EXHIBIT D

An official website of the United States government
Here's how you know

Synthetic construct FT_Tv7_d gene, complete cds

GenBank: MH043115.1

FASTA Graphics

Go to:

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  AUTHORS   Larue,C.T.
  TITLE     Development of Robust FOP and Synthetic Auxin Herbicide Tolerance
            Traits for GE Crops
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 888)
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  TITLE     Direct Submission
  JOURNAL   Submitted (12-MAR-2018) CV, Monsanto Company, 700 Chesterfield PKWY
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EXHIBIT E

RECEIVED
By USDA APHIS BRS Document Control Officer at 2:18 pm, Dec 11, 2019

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MONSANTO

Petition for the Determination of Nonregulated Status for Dicamba, Glufosinate, Quizalofop and 2,4-Dichlorophenoxyacetic Acid Tolerant MON 87429 Maize with Tissue-Specific Glyphosate Tolerance Facilitating the Production of Hybrid Maize Seed

The undersigned submits this petition under 7 CFR § 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR Part 340

June 27, 2019
(Revised December 10, 2019)

OECD Unique Identifier: MON-87429-9

Monsanto Petition number: CR279-19U4

Submitted by:

David Wilson, Ph.D.

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RELEASE OF INFORMATION

Monsanto[1] is submitting the information in this petition for review by the USDA as part of the regulatory process. Monsanto understands that the USDA complies with the provisions of the Freedom of Information Act (FOIA). In the event the USDA receives a FOIA request, pursuant to 5 U.S.C., § 552, and 7 CFR Part 1, covering all or some of the information in this petition, Monsanto expects that, in advance of the release of the document(s), USDA will provide Monsanto with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g., responsiveness, confidentiality, and/or competitive concerns. Monsanto understands that a CBI-deleted copy of this information may be made available to the public in a reading room and upon individual request as part of a public comment period. Monsanto also understands that when deemed complete, a copy of the petition may be posted to the USDA-APHIS APHIS-BRS website or other U.S. government websites (e.g., www.regulations.gov). Except in accordance with the foregoing, Monsanto does not authorize the release, publication or other distribution of this information without Monsanto's prior notice and consent.

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[1] Monsanto Company remains a legal entity holding registrations, permits and other approvals, but it is wholly owned by Bayer. We will let you know about any reorganization of legal entities, including transfer of authorizations/registrations from Monsanto and/or address updates. The names of entities holding registrations should not have any bearing on our approvals. These changes are for legal administrative reasons and have no influence on the quality of the end-use products, grain or seeds and they have no consequences for existing safety evaluations.

CBI Deleted

CERTIFICATION

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioner that are unfavorable to the petition.

David Wilson, Ph.D.
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EXECUTIVE SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S.

APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company is submitting this request to APHIS for a determination of nonregulated status for the new biotechnology-derived maize product, MON 87429, any progeny derived from crosses between MON 87429 and conventional maize, and any progeny derived from crosses of MON 87429 with biotechnology-derived maize that have previously been granted nonregulated status under 7 CFR Part 340.

Product Description

Monsanto Company has developed herbicide tolerant MON 87429 maize, which is tolerant to the herbicides dicamba, glufosinate, aryloxyphenoxypropionate (AOPP) acetyl coenzyme A carboxylase (ACCase) inhibitors (so called "FOPs" herbicides such as quizalofop) and 2,4-dichlorophenoxyacetic acid (2,4-D). In addition, it provides tissue-specific glyphosate tolerance to facilitate the production of hybrid maize seeds. MON 87429 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide, the phosphinothricin-N-acetyltransferase (*pat*) gene from *Streptomyces viridochromogenes* that expresses the PAT protein to confer tolerance to glufosinate herbicide and the *ft_t* gene, a modified version of the R-2,4-dichlorophenoxypropionate dioxygenase *(Rdpa*) gene from *Sphingobium herbicidovorans*, that expresses a FOPs and 2,4-D dioxygenase protein (FT_T) that confers tolerance to quizalofop and 2,4-D herbicides. MON 87429 maize also produces the 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) to confer tolerance to glyphosate for use in hybrid seed production. MON 87429 maize utilizes an endogenous maize regulatory element to target CP4 EPSPS mRNA for degradation in tassel tissues, resulting in reduced CP4 EPSPS protein expression in pollen. Appropriately timed glyphosate applications produce a non-viable pollen phenotype and allow for desirable cross pollinations to be made in maize without using mechanical or manual detasseling methods to control self-pollination in female inbred parents.

Tissue-specific expression of CP4 EPSPS protein in MON 87429, allowing for glyphosate induced non-viable pollen phenotype, is the second generation of Monsanto's Roundup® Hybridization System (RHS) for hybrid seed production. The first-generation RHS event, MON 87427 maize, was deregulated in 2013 (USDA-APHIS Petition #10-281-01p). The second-generation RHS trait in MON 87429 allows inbred MON 87429 lines, treated with glyphosate at the appropriate timings, to serve as a female parent in the production of hybrid

seed. Female inbred MON 87429 lines receive two glyphosate applications at vegetative growth stages ranging from V8 to V13 that correspond to the time when immature male reproductive tissues are forming. Treatment with glyphosate at these stages results in the intended non-viable pollen phenotype in MON 87429 inbred lines due to tissue-specific glyphosate sensitivity in the immature male reproductive tissue. In hybrid maize production systems, treatment of female inbred MON 87429 plants with glyphosate during the time immature male reproductive tissue is developing will inhibit self-pollination. MON 87429 female inbreds instead will be pollinated by a desired male pollen donor inbred grown in close proximity that contains a deregulated glyphosate tolerance trait, such as NK603. This cross-pollination results in hybrid offspring (e.g., MON 87429 × NK603) with full plant tolerance to glyphosate, both in vegetative and reproductive tissues, as well as tolerance to dicamba, glufosinate, quizalofop, and 2,4-D herbicides.

The RHS trait in MON 87429 maize offers the same benefits to hybrid maize seed production as the RHS trait in MON 87427, described in detail in USDA-APHIS Petition #10-281-01p. Briefly, these benefits include enabling hybrid seed producers to discontinue the practice of manually or mechanically detasseling female inbred plants in their production field, which must occur during a critical 3-4 day time period of maize tassel development, which can be influenced by changes in weather (e.g., extreme heat). The ability to treat MON 87429 maize female inbreds with glyphosate (between V8 to V13), in place of detasseling, provides flexibility to hybrid maize seed producers as well as reduces the cost of hybrid seed production by removing the reliance on costly, labor intensive manual/mechanical detasseling. An additional benefit of including the RHS trait in MON 87429 maize, along with dicamba-, glufosinate-, and quizalofop- and 2,4-D-tolerance traits, is the reduction in the number of trait loci that would otherwise need to be managed and combined, via traditional breeding methods.

The MON 87429 event confers glyphosate tolerance in specific plant tissues (i.e., not in tassels) and will be used to facilitate the production of hybrid seed. MON 87429 is not intended to be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated events that confer full-plant glyphosate tolerance (e.g., NK603). MON 87429 maize combined with the glyphosate-tolerant maize system through traditional breeding will provide growers: 1) an opportunity for an efficient, effective weed management system for hard-to-control and herbicide-resistant weeds; 2) a flexible system with multiple herbicide sites-of-action for in-crop application in current maize production systems; 3) an opportunity to delay selection for further resistance to glyphosate and other herbicides that are important in crop production; 4) excellent crop tolerance to dicamba, glufosinate, quizalofop, 2,4-D and glyphosate; and 5) additional weed management tools to enhance weed management systems necessary to maintain or improve maize yield and quality to meet the growing needs of the food, feed, and industrial markets.

MON 87429 maize will offer growers multiple choices for effective weed management including tough-to-control and herbicide-resistant broadleaf and grass weeds. The flexibility to use combinations of dicamba, glufosinate, 2,4-D, quizalofop and glyphosate herbicides representing multiple sites-of-action provides an effective weed management system for maize production. Dicamba provides effective control of over 95 annual and biennial broadleaf weed species, approximately 50 perennial broadleaf species and control or suppression of over 50 woody plant species. Glufosinate, a broad-spectrum contact herbicide, provides effective control of

approximately 70 annual broadleaf weed species, over 30 grass weeds and control or suppression of over 30 biennial and perennial grass and broadleaf weed species. Quizalofop, a selective postemergence herbicide, provides effective control of approximately 35 annual and perennial grass weeds including glyphosate-resistant grasses. 2,4-D provides effective control of over 70 annual broadleaf weed species, and approximately 30 perennial broadleaf species. Glyphosate provides control of approximately 100 annual weed species (grass and broadleaf), over 60 perennial weed species (grass and broadleaf) and control or suppression of approximately 65 woody brush, trees and vines. Additionally, dicamba, glufosinate, and 2,4-D individually or in certain combinations provide control of herbicide-resistant weeds, including glyphosate-resistant biotypes of Palmer amaranth (*Amaranthus palmeri*), marestail (*Conyza canadensis*), common ragweed (*Ambrosia artemisiifolia*), giant ragweed (*Ambrosia trifida*) and waterhemp (*Amaranthus tuberculatus*).

Data and Information Presented Confirms the Lack of Plant Pest Potential and the Food and Feed Safety of MON 87429 Compared to Conventional Maize

The data and information presented in this petition demonstrate MON 87429 is agronomically and phenotypically comparable to commercially cultivated maize, with the exception of the introduced traits. Moreover, the data and information presented demonstrate MON 87429 is not expected to pose an increased plant pest risk, including weediness, compared to commercially cultivated maize. The food, feed, and environmental safety of MON 87429 was confirmed based on multiple, well-established lines of evidence:

- Maize is a familiar crop that does not possess any of the attributes commonly associated with weeds and has a history of safe consumption. The conventional control used for the transformation process was included in studies to serve as an appropriate basis of comparison for MON 87429.

- A detailed molecular characterization of the inserted DNA demonstrates a single, intact copy of the T-DNA insert in a single locus within the maize genome.

- Extensive evaluation of the FT_T protein and previous assessments of the DMO, PAT and CP4 EPSPS proteins expressed in MON 87429, confirm they are unlikely to be toxins or allergens.

- An extensive evaluation of MON 87429 phenotypic and agronomic characteristics and environmental interactions demonstrates MON 87429 has no increased plant pest risk compared to conventional maize.

- An assessment of potential impact to non-target organisms (NTOs) including organisms beneficial to agriculture and endangered species indicates that MON 87429 is not expected to have an adverse effect on other organisms compared to conventional maize under normal agricultural practices.

- Evaluation of the agronomic and phenotypic characteristics of MON 87429, using current cultivation and management practices, leads to the conclusion that deregulation of MON 87429 is not expected to have an adverse effect on maize agronomic practices.

Maize is a Familiar Crop Lacking Weedy Characteristics

Maize is grown extensively throughout the world and is the largest cultivated grain crop followed by wheat (*Triticum* sp.) and rice (*Oryza sativa* L.) in total global production. In the U.S., maize is grown in almost all states and is the largest crop grown in terms of net value. Maize has been studied extensively, and the initial steps in its domestication can be traced back to approximately 9,000 years ago in southern Mexico. Although grown extensively throughout the world, maize is not considered a threat to invade natural or agricultural ecosystems because it does not establish self-sustaining populations outside of cultivation. This lack of weediness may reflect its poor competitive ability, lack of seed dormancy, and barriers to seed dispersal, as maize cobs retain seed and are covered in a husk. Several other characteristics common in weeds, such as rapid flowering following emergence, are lacking in maize. Traits often associated with weediness are typically not selected for during domestication and subsequent breeding and selection, and similarly, the history of maize breeding and production in the U.S. does not indicate there are any changes in the characteristics of maize that would increase the weediness of the crop. Although maize seed can overwinter in a rotation with soybeans or other crops, mechanical and chemical measures are routinely used to control maize volunteers. Maize is not sexually compatible with plant species occurring in the U.S. other than teosinte, an introduced wild relative. However, gene introgression from maize into teosinte is unlikely in the U.S. due to barriers to crossing, including morphological and developmental differences and limited geographical distribution of teosinte populations.

Conventional Maize LH244 is an Appropriate Comparator to MON 87429

Conventional control materials developed for use as comparators in safety assessment studies were based on the type of study conducted and the genetic background of the test material. The conventional control materials included the original transformation line (LH244) and LH244 crossed to a conventional line (HCL617) to create F1 starting control materials. LH244 was used as the control in molecular characterization studies. LH244 × HCL617 was used as the control in compositional analysis studies and in phenotypic, agronomic and environmental interactions assessments. Where appropriate, commercial hybrid maize materials (reference hybrids) were also used to establish a range of variability or responses representative of commercial maize in the U.S.

Molecular Characterization Verified the Integrity and Stability of the Inserted DNA in MON 87429

MON 87429 was produced by *Agrobacterium* mediated-transformation of maize tissue using the T-DNA transformation vector PV-ZMHT519224. This plasmid vector contains a single transfer DNA (T-DNA), that is delineated by Right and Left Border regions. The T-DNA contains the *pat*, *dmo, ft_t*, and *cp4 epsps* expression cassettes. Following transformation, traditional breeding, segregation, selection and screening were used to isolate those plants that contain the *pat*, *dmo*, *ft_t*, and *cp4 epsps* expression cassettes and do not contain any plasmid backbone sequences.

Characterization of the DNA insert in MON 87429 was conducted using a combination of sequencing, polymerase chain reaction (PCR), and bioinformatics. The results of this

characterization demonstrate that MON 87429 contains one copy of the intended T-DNA containing the *pat*, *dmo*, *ft_t*, and *cp4 epsps* expression cassettes that is stably inherited over multiple generations and segregates according to Mendelian principles. These conclusions are based on several lines of evidence:

- Molecular characterization of MON 87429 by Next Generation Sequencing (NGS) demonstrated that MON 87429 contains a single intended DNA insert. These whole-genome analyses provided a comprehensive assessment of MON 87429 to determine the presence and identity of sequences derived from PV-ZMHT519224 and demonstrated that MON 87429 contains a single T-DNA insert with no detectable plasmid backbone sequences.

- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) performed on MON 87429 was used to determine the complete sequence of the single DNA insert from PV-ZMHT519224, the adjacent flanking genomic DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the sequence and organization of the DNA is identical to the corresponding region in the PV-ZMHT519224 T-DNA. Furthermore, the genomic organization at the insertion site in MON 87429 was assessed by comparing the sequences flanking the T-DNA insert in MON 87429 to the sequence of the insertion site in conventional maize. This analysis determined that 54 bases were deleted upon T-DNA integration. There also was a 29 base insertion in the MON 87429 5′ flanking sequence and a 31 base insertion in the MON 87429 3′ flanking sequence.

- Generational stability analysis by Next Generation Sequencing (NGS) demonstrated that the single PV-ZMHT519224 T-DNA insert in MON 87429 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 87429.

- Segregation analysis corroborates the insert stability demonstrated by NGS and independently establishes the nature of the T-DNA as a single chromosomal locus that shows an expected pattern of inheritance.

Taken together, the characterization of the genetic modification in MON 87429 demonstrates that a single copy of the intended T-DNA was stably integrated at a single locus of the maize genome and that no plasmid backbone sequences are present in MON 87429.

In addition, MON 87429 maize utilizes an endogenous maize regulatory element (a target sequence for endogenous small interfering RNAs (siRNAs)) to target CP4 EPSPS mRNA for degradation in tassel tissues. A focused study on the MON 87429 siRNA Target Sequence demonstrated the absence of unintended effects on endogenous gene regulation.

Data Confirm DMO, PAT, CP4 EPSPS and FT T Protein Safety

MON 87429 contains a *dmo* expression cassette that expresses a single MON 87429 DMO precursor protein that is post-translationally processed during the chloroplast targeting process into two forms of the DMO protein; referred to as MON 87429 DMO+1 and MON 87429 DMO+0. MON 87429 DMO+1 is identical to MON 87429 DMO+0 with the exception that it contains an additional amino acid on the N-terminus, a cysteine residue, derived from the alternative processing of the chloroplast transit peptide APG6. Given this degree of similarity, the term MON 87429 DMO protein will be used hereafter to refer to both forms of the protein collectively and distinctions will only be made where necessary. DMO proteins highly similar to those produced in MON 87429 are also present in MON 88701 cotton, MON 87708 soybean and MON 87419 maize which were deregulated by USDA-APHIS in 2015, 2015 and 2016, respectively. MON 88701 cotton, MON 87708 soybean and MON 87419 maize also completed FDA consultation in 2013, 2011 and 2016, respectively, where it was demonstrated that food and feed derived from these crops are not materially different than the respective conventional crops. Data, demonstrating the safety of DMO, were reviewed by U.S. agencies in accordance with the review responsibilities under the Coordinated Framework, resulting in full authorization of these products in the U.S. The safety of DMO protein has been favorably assessed following extensive reviews by regulatory agencies in at least 12 different countries. Although there are minor differences in amino acid sequence, the DMO proteins expressed in MON 87429 are identical to previously reviewed DMO proteins in terms of structure of the catalytic site, function, immunoreactivity, and substrate specificity. Thus, prior safety assessments of DMO proteins are applicable to the DMO protein expressed in MON 87429.

The PAT protein in MON 87429 has the same sequence as PAT protein produced in several other commercially available crops that have been reviewed by USDA and previously deregulated (e.g., T25, DAS-59122-7 and TC1507 maize and A2704-12 and A5547-127 soybean). The safety of PAT proteins has been confirmed following extensive reviews by regulatory agencies in at least 15 different countries for more than 30 biotechnology-derived events in several different crop species (e.g., maize, soybean, cotton, canola and sugar beet). The lack of any documented reports of adverse effects of PAT-containing crops since their commercial introduction further confirms the safety of the PAT protein. The amino acid sequence of the PAT protein expressed in MON 87429 is identical to the wild type PAT protein encoded by *S. viridochromogenes* except for the first methionine, which is removed due to co-translational processing in MON 87429. N-terminal methionine cleavage is common and naturally occurs in the vast majority of proteins. Thus, prior safety assessments of PAT proteins are applicable to the PAT protein expressed in MON 87429.

The CP4 EPSPS protein in MON 87429 has the same sequence as CP4 EPSPS proteins produced in several other commercially available crops that have been reviewed by USDA and previously deregulated (e.g., 40-3-2 and MON 89788 soybean, NK603, MON 87427 and MON 88017 maize, RT73 canola, H7-1 sugar beet, 1445 and MON 88913 cotton, and J101 and J163 alfalfa). The safety and mode-of-action of CP4 EPSPS proteins is well documented and is the subject of numerous publications. Additionally, in 1996 the U.S. EPA established an exemption from the requirement of a tolerance for residues of the plant pesticide inert ingredient CP4 EPSPS and the genetic material necessary for its production in all plants (40 CFR § 174.523, redesignated from § 180.1174, effective April 25, 2007). The safety of the CP4 EPSPS protein as expressed in

MON 88017 and MON 87427 maize has also been reviewed and approved in at least 12 countries. Thus, prior safety assessments of CP4 EPSPS protein are applicable to the CP4 EPSPS protein expressed in MON 87429.

A multistep approach was used to characterize and assess the safety of the FT_T protein expressed in MON 87429 resulting from the genetic modification. The expression level of the FT_T protein in selected tissues of MON 87429 was determined and potential for exposure to humans and animals was assessed. In addition, the donor species for the FT_T protein coding sequences, *Sphingobium* species, are ubiquitous in the environment and are not commonly known for human or animal pathogenicity or allergenicity. Bioinformatics analysis determined that the FT_T protein lacks structural similarity to known allergens, gliadins, glutenins, or protein toxins. The FT_T protein is rapidly digested in pepsin and pancreatin and demonstrates no acute oral toxicity in mice at a dose level that far exceeds anticipated exposure by humans and animals. Hence, the consumption of the FT_T protein from MON 87429 or its progeny poses no meaningful risk to human and animal health.

MON 87429 is Compositionally Equivalent to Conventional Maize

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Maize is not known to have any endogenous toxicants or antinutrients associated with overall plant pest potential. For MON 87429, the introduced proteins, DMO, PAT, CP4 EPSPS and FT_T, confer herbicide tolerance and lack catalytic activity that is intended to or expected to affect the plant's metabolism. Given the nature of these introduced traits and the overall lack of meaningful unintended compositional characteristics observed for biotechnology-derived products characterized to date, compositional changes that would affect the levels of nutritional components in MON 87429 maize were not expected.

Monsanto is currently in consultation with the Food and Drug Administration following their policy, "Foods Derived from New Plant Varieties," on the food and feed safety of MON 87429 maize (Submitted 05-Feb-2019). Composition data for 25 components including major nutrients in grain and forage and anti-nutrients in grain was submitted to FDA as part of the voluntary food/feed safety and nutritional assessment for MON 87429 maize. The results of the compositional assessment found that there were no biologically meaningful differences between MON 87429 and conventional control and support the conclusion that MON 87429 maize is compositionally equivalent to the conventional control.

MON 87429 Does Not Change Maize Plant Pest Potential or Environmental Interactions

Plant pest potential of a biotechnology-derived crop is assessed from the basis of familiarity that the USDA recognizes as an important underlying concept in risk assessment. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant hybrid or variety whose biological properties and plant pest potential are well known. Familiarity considers the biology of the plant, the introduced trait, the receiving environment, and the interactions among these factors. This provides a basis for comparative risk assessment between a biotechnology-derived plant and the conventional control. Thus, the

phenotypic, agronomic, and environmental interactions assessment of MON 87429 included the genetically similar conventional control as a comparator. This evaluation used a weight of evidence approach and considered statistical differences between MON 87429 and the conventional control with respect to reproducibility, magnitude, and directionality. Comparison to a range of commercial references grown concurrently established the range of natural variability for maize and provided a context from which to further evaluate any statistical differences. Assessments included seed germination and dormancy characteristics and pollen characteristics in the laboratory as well as phenotypic and agronomic characteristics and plant responses to abiotic stressors, diseases, and arthropod pests in the field. Results from the phenotypic, agronomic, and environmental interactions assessment indicated that MON 87429 does not possess enhanced weediness characteristics, increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests, or characteristics that would confer an increased plant pest risk compared to conventional maize.

MON 87429 Will Not Negatively Affect Non-Target Organisms (NTOs) Including Those Beneficial to Agriculture

An evaluation of the impacts of MON 87429 on non-target organisms (NTOs) is a component of the plant pest risk assessment. Because MON 87429 does not possess pesticidal activity, all organisms that interact with MON 87429 are considered to be NTOs. Data from the 2017 U.S. phenotypic and agronomic studies, including observational data on plant response to abiotic stressors, diseases, and arthropod pests, were collected for MON 87429 and conventional controls. Results from these studies support conclusions that MON 87429 is unlikely to adversely affect NTOs.

The DMO, PAT, and CP4 EPSPS proteins have been assessed in multiple products by USDA-APHIS and U.S. FDA in the past years. DMO protein is produced in both MON 87708 soybean, MON 88701 cotton and MON 87419 maize that were granted nonregulated status by USDA-APHIS. Starting in 1996 with Bayer's T25 maize, numerous glufosinate tolerant crops (canola, cotton, maize, soybean, sugar beet) containing PAT proteins have been granted nonregulated status by USDA-APHIS. CP4 EPSPS protein is also produced in numerous other glyphosate tolerant crops (soybean, maize, cotton, sugar beet, canola, and alfalfa) that have been granted nonregulated status by USDA-APHIS. Assessment of the FT_T proteins non-toxic mode-of-action (MOA), lack of acute oral toxicity, and lack of impact on plant pest potential supports a conclusion that the FT_T protein will not have adverse impacts to NTOs. In addition, after either extensive testing and/or wide scale commercial cultivation, in no instance have adverse impacts to NTOs been associated with exposure to DMO, PAT, or CP4 EPSPS proteins from these biotechnology-derived crops.

The biochemical information and experimental data for evaluation of MON 87429 included mode of action, molecular characterization, DMO, PAT, CP4 EPSPS and FT_T protein safety assessment, establishment of compositional equivalence to conventional maize, data from the environmental interactions assessment, and demonstration of agronomic and phenotypic equivalence to conventional maize. Taken together, these data support the conclusion that MON 87429 has no plausible mechanism for harm to NTOs, nor does it pose an additional risk to organisms beneficial to agriculture or threatened and endangered species compared to conventional maize.

Deregulation of MON 87429 is Not Expected to Have Effects on Maize Agronomic Practices

An assessment of current maize agronomic practices was conducted to determine whether the cultivation of MON 87429 has the potential to impact current maize agronomic practices. Maize fields are typically highly managed areas that are dedicated to grain and/or forage production. MON 87429 has been developed to offer maize growers multiple choices for effective weed management including tough to control and herbicide resistant broadleaf and grass weeds. The combination of dicamba, glufosinate, quizalofop and 2,4-D tolerance offers multiple herbicide sites-of-actions and provides an effective weed management system for maize production in the U.S. Dicamba, glufosinate, quizalofop, and 2,4-D herbicides are currently labeled for preplant and postemergence applications in maize. In support of Monsanto's third generation herbicide tolerant MON 87419 maize, deregulated by USDA in 2016, Monsanto requested that EPA allow the 0.5 lb a.e./ac postemergence application window for dicamba to be extended from V5 to V8 growth stage or 36-inch height of maize, whichever occurs first, without reducing the application rate of dicamba (U.S. EPA, 2019). The combined (pre- and post-emergence) maximum annual application rate of dicamba in MON 87419 maize would be 2.0 lbs. a.e. dicamba per acre per year, increased from the current maximum annual application rate of 0.75 lbs. a.e. in conventional maize. Pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of MON 87419. Glufosinate, quizalofop and 2,4-D, are currently labeled for preplant applications on conventional and herbicide-tolerant maize hybrids and for in-crop postemergence applications on herbicide-tolerant hybrids. Use of glufosinate, quizalofop and 2,4-D over the top of MON 87429 will follow the current labeled use patterns of these individual herbicides. The MON 87429 event confers glyphosate tolerance in specific plant tissues (i.e., not in tassels) and will be used to facilitate the production of hybrid seed. MON 87429 is not intended to be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated events that confer full-plant glyphosate tolerance (e.g., NK603). The introduction of MON 87429 stacked with a deregulated glyphosate tolerance trait will follow the current labeled use patterns of glyphosate herbicide. The introduction of MON 87429 maize is not expected to have adverse impacts on current agronomic, cultivation and management practices for maize. No meaningful changes are anticipated in crop rotations, tillage practices, planting practices, fertility management, weed and disease management, and volunteer management from the introduction of MON 87429.

Conclusion

Based on the data and information presented in this petition, it is concluded that MON 87429 is unlikely to pose a plant pest risk. Therefore, Monsanto Company requests a determination from USDA-APHIS that MON 87429 and any progeny derived from crosses between MON 87429 and conventional maize or deregulated biotechnology-derived maize be granted nonregulated status under 7 CFR Part 340.

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ABBREVIATIONS AND DEFINITIONS[2]

αKG	alpha-ketoglutarate
2,4-D	2,4-Dichlorophenoxyacetic Acid
ACCase	Acetyl Coenzyme A Carboxylase
APHIS	Animal and Plant Health Inspection Service
aroA	5-enolpyruvylshikimate-3-phosphate synthetase gene from *Agrobacterium* sp. strain CP4
CAB	Chlorophyll a/b-Binding
CaMV	Cauliflower Mosaic Virus
CFR	Code of Federal Regulations
CP4 EPSPS	*Agrobacterium tumefaciens* strain CP4, 5-enolpyruvlshikimate-3-phosphate synthase protein
CTP	Chloroplast Transit Peptide
CTAB	Hexadecyltrimethylammonium Bromide
CV	Column Volume
BIO	Biotechnology Industry Organization
BRS	Biotechnology Regulatory Service
DAP	Days After Planting
DCSA	3,6-Dichlorosalycilic Acid
DMO	Dicamba Mono-Oxygenase
DNA	Deoxyribonucleic Acid
dw	Dry Weight
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELN	Electronic Lab Notebook
EPA	Environmental Protection Agency
E-score	Expectation Score
ETS	Excellence Through Stewardship
FDA	Food and Drug Administration (U.S.)
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FOIA	Freedom of Information Act
FOPs	aryloxyphenoxypropionate acetyl coenzyme A carboxylase inhibitor herbicides such as quizalofop
FT_T	FOPs and 2,4-D Dioxygenase Protein
GLP	Good Laboratory Practice
IAC	Immunoaffinity Chromatography
IAM	Iodoacetamide
kb	Kilobase
LOQ	Limit of Quantitation

[2] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

ABBREVIATIONS AND DEFINITIONS (continued)

MBu	Million bushels
Mdh	malate dehydrogenase gene from *Arabidopsis thaliana*
MOA	Mode of Action
mRNA	Messenger RNA
mts-siRNA	Male Tissue Specific Small Interfering RNA
MW	Molecular Weight
NGS	Next Generation Sequencing
OECD	Organization for Economic Co-operation and Development
OPA	o-phthalaldehyde
ORF	Open Reading Frame
OSL	Over Season Leaf
PAT	Phosphinothricin N–Acetyltransferase
PCR	Polymerase Chain Reaction
RdpA	R-2,4-Dichlorophenoxypropionate Dioxygenase
RHS	Roundup® Hybridization System
S3P	Shikimate-3phoshate
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SE	Standard Error
ShkG	5-enolpyruvylshikimate-3-phosphate synthase from *Arabidopsis thaliana*
siRNA	Small Interfering RNA
SOA	Site-of-action
SOP	Standard Operating Procedure
µg/g	Microgram per Gram
USDA	United States Department of Agriculture
WT	Wildtype

I. RATIONALE FOR THE DEVELOPMENT OF MON 87429

I.A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR § 340.6

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-2a24, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. APHIS regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and no longer should be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company is submitting this request to APHIS for a determination of nonregulated status for the new biotechnology-derived maize product, MON 87429, any progeny derived from crosses between MON 87429 and conventional maize, and any progeny derived from crosses of MON 87429 with biotechnology-derived maize that have previously been granted nonregulated status under 7 CFR Part 340.

I.B. Rationale for the Development of Herbicide Tolerant Maize MON 87429

Maize (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. Planted maize acres in the U.S. have ranged between 88 to 94 million acres from 2015 to 2018 (USDA-NASS, 2018a). Annual and perennial weeds are considered to be the greatest pest problem in corn production (Aref and Pike, 1998). Weeds compete with maize for water, nutrients, and light resulting in substantial yield losses when left uncontrolled. Weed species in maize vary from region to region and from state to state. Economic thresholds for controlling weeds in maize require some form of weed management practice on all maize acreage. Weed management practices include mechanical tillage, crop rotations, cultural practices, and herbicide application. Numerous selective herbicides are available for preplant, preemergence, and postemergence control of annual and perennial weeds in maize. Approximately 98% of the maize acreage in the U.S. receives an herbicide application (USDA-NASS, 2019b).

MON 87429 maize will offer growers multiple choices for effective weed management including tough-to-control and herbicide-resistant broadleaf and grass weeds. The flexibility to use dicamba, glufosinate, quizalofop, 2,4-D and glyphosate and/or combinations of these five herbicides representing multiple sites-of-action will provide an effective weed management system for maize production. Dicamba provides effective control of over 95 annual and biennial broadleaf weed species, approximately 50 perennial broadleaf species and control or suppression of over 50 woody plant species. Glufosinate, a broad-spectrum contact herbicide, provides effective control of approximately 70 annual broadleaf weed species and over 30 grass weed species and control or suppression of over 30 biennial and perennial grass and broadleaf weed

species. Quizalofop, a selective postemergence herbicide, provides effective control of approximately 35 annual and perennial grass weed species including glyphosate-resistant grasses. 2,4-D provides effective control of over 70 annual and biennial broadleaf weed species, and approximately 30 perennial broadleaf weed species. Glyphosate provides control of approximately 100 annual weed species (grass and broadleaf), over 60 perennial weed species (grass and broadleaf) and control or suppression of approximately 65 woody brush, trees and vines. Additionally, dicamba, glufosinate, and 2,4-D individually or in certain combinations provide control of herbicide-resistant weeds, including glyphosate-resistant biotypes of Palmer amaranth (*Amaranthus palmeri*), marestail (*Conyza canadensis*), common ragweed (*Ambrosia artemisiifolia*), giant ragweed (*Ambrosia trifida*), johnson grass (*Sorghum halepense)* and waterhemp (*Amaranthus tuberculatus*).

The MON 87429 event confers glyphosate tolerance in specific plant tissues (i.e., not in tassels) and will be used to facilitate the production of hybrid seed. MON 87429 is not intended to be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated events that confer full-plant glyphosate tolerance (e.g., NK603). MON 87429 maize combined with glyphosate-tolerant maize through traditional breeding will provide: 1) an opportunity for an efficient, effective weed management system for hard-to-control and herbicide-resistant weeds; 2) a flexible system with multiple herbicide sites-of-action for in-crop application in current maize production systems; 3) an opportunity to delay selection for further resistance to glyphosate and other herbicides that are important in crop production; and 4) excellent crop tolerance to dicamba, glufosinate, quizalofop, 2,4-D and glyphosate. These enhanced weed management tools will maintain or improve maize yield and quality to meet the growing needs of the food, feed, and industrial markets.

Maize differs from other major U.S. crops, such as soybean or cotton, in that it is typically planted as a hybrid, and maize hybrids are utilized on nearly all maize production acres currently planted in the U.S. The seed supply used to plant the U.S. maize acreage is produced via hybrid seed production. The volume of seed planted in the U.S., to produce hybrid maize seed, has increased from 22.45 million bushels (MBu) planted in 2000 to 30.90 MBu planted in 2018 (USDA-ERS, 2018a). Hybrid seed production is accomplished through the combining of genetic material from one inbred parent with that of the other inbred parent. Specifically, pollen from the tassel (male flower) of the male parent is used to fertilize the ear (female flower) of the female parent. One challenge inherent to the production of hybrid maize seed is that the female parent produces pollen at the same time as the male parent. Therefore, pollen from the female parent must be removed or eliminated in order to assure uni-directional transfer of genetic material, via pollen, only from the male parent to the female parent. In the past, removal or elimination of the pollen from the female parent required deployment of costly, labor intensive and time-critical methods such as manual or mechanical detasseling, or maintenance of Cytoplasmic Male Sterile lines, to ensure quality hybrid seed was produced. These hybrid seed production methods are further detailed in MON 87427 USDA-APHIS Petition #10-281-01p (p. 128), Monsanto's first-generation

Roundup® Hybridization System (RHS) for hybrid seed production, which was assessed and deregulated by USDA-APHIS in 2013.

Tissue-specific expression of CP4 EPSPS protein in MON 87429, allowing for glyphosate induced non-viable pollen phenotype, is the second-generation of Monsanto's Roundup® Hybridization System (RHS) for hybrid seed production. The first-generation RHS event, MON 87427 maize, was assessed by USDA-APHIS and deregulated in 2013 (USDA-APHIS Petition #10-281-01p). The second-generation RHS trait in MON 87429 allows inbred MON 87429 lines, treated with glyphosate at the appropriate timings, to serve as a female parent in the production of hybrid seed, similar to previously deregulated MON 87427 maize. Female inbred MON 87429 lines receive two glyphosate applications at vegetative growth stages ranging from V8 to V13, that correspond to the time when immature male tissues are forming. Treatment with glyphosate during this time period, when male tissue is forming, results in the intended non-viable pollen phenotype due to tissue-specific glyphosate sensitivity in the immature male tissue (See Section IV.G).

To produce commercial hybrid maize seed with whole plant glyphosate tolerance using the RHS hybrid seed production system, female inbred MON 87429 plants treated with glyphosate during immature male tissue development will be pollinated by pollen donor plants (male inbred) that contain a deregulated glyphosate tolerance trait (e.g., NK603). The male inbred plants will be cultivated in proximity to the female inbred MON 87429 plants and will not be impacted by the RHS-required glyphosate applications, due to full glyphosate tolerance in all tissues. This cross-pollination results in hybrid offspring (e.g., MON 87429 × NK603) with full tolerance to glyphosate in both vegetative and reproductive tissues, as well as tolerance to dicamba, glufosinate, quizalofop, and 2,4-D herbicides. For weed control in hybrid seed production fields with MON 87429 maize inbred used as female parent, in-crop applications of glyphosate may be made at the same rates and timings as directed on glyphosate agricultural product labels for other glyphosate tolerant maize events.

The RHS trait in MON 87429 maize offers the same benefits to hybrid maize seed production as the RHS trait in MON 87427, described in detail in USDA-APHIS Petition #10-281-01p (p. 5). Briefly, these benefits include enabling hybrid seed producers to discontinue the practice of manually or mechanically detasseling female inbred plants in their production field, which must occur during a critical 3-4 day time period of maize tassel development, which can be influenced by changes in weather (e.g., extreme heat). The ability to treat MON 87429 maize female inbreds with glyphosate (between V8 to V13), in place of detasseling, provides flexibility to hybrid maize seed producers as well as reduces the cost of hybrid seed production by removing the reliance on costly, labor intensive manual/mechanical detasseling. An additional benefit of including the RHS trait in MON 87429 maize, along with dicamba-, glufosinate-, and quizalofop- and 2,4-D-tolerance traits, is the reduction in the number of trait loci that would otherwise need to be managed and combined, via traditional breeding methods.

I.C. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology (CFR) (USDA-APHIS, 1986), the responsibility for regulatory oversight of biotechnology-derived crops falls primarily on three U.S. agencies: U.S. Food and Drug Administration (FDA), the United States Department of Agriculture (USDA), and in the case of plant incorporated protectants (PIPs), the Environmental Protection Agency (EPA). Deregulation of MON 87429 by USDA constitutes only one component of the overall regulatory oversight and review of this product. As a practical matter, MON 87429 cannot be released and marketed until FDA and USDA have completed their reviews and assessments under their respective jurisdictions. As MON 87429 does not contain a PIP, no submission on MON 87429 will be made to the EPA.

I.C.1. Submission to FDA

MON 87429 falls within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (U.S. FDA, 1992). In compliance with this policy, Monsanto has initiated a consultation with the FDA, identified under BNF No. 000173. A food/feed safety and nutritional assessment summary document was submitted to the FDA in February 2019.

I.C.2. Related Submissions to U.S. EPA

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA; 7 U.S.C. §136(u)), are subject to regulation by U.S. EPA. When registering the use of herbicides, U.S. EPA performs a thorough risk assessment including acute, chronic, and aggregate risks for the U.S. population, including setting safe limits for pesticide residues including parent molecules and metabolites. In each case a determination of safety is made that no harm will result to the general population or to infants and children from the aggregate exposure of the herbicide.

MON 87429 provides improved crop tolerance to dicamba, and the ability to extend use of dicamba through the V8 growth stage will provide more effective preemergence and postemergence control of problem weed species compared to currently labeled applications of dicamba in conventional maize hybrids. In support of Monsanto's third generation herbicide tolerant product MON 87419 maize, Monsanto requested that EPA allow the 0.5 lb a.e./ac postemergence application window for dicamba to be extended from V5 to V8 growth stage or 36-inch height of maize, whichever occurs first (U.S. EPA, 2019). The combined (pre- and post-emergence) maximum annual application rate of dicamba in MON 87419 maize would be 2.0 lbs. a.e. dicamba per acre per year, increased from the current maximum annual application rate of 0.75 lbs. a.e. in conventional maize. Pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of MON 87419.

Glufosinate use over the top of MON 87429 will not change from current approved use pattern of glufosinate[3]. Glufosinate is labeled for preplant applications on conventional and herbicide-tolerant maize hybrids and for in-crop postemergence applications on glufosinate-tolerant hybrids only.

U.S. EPA has registered the use of 2,4-D on maize[4] up to 1 lb. preemergence and one to two applications of up to 1 lb. between emergence and V8 or 30 inches whichever occurs first. Monsanto, or a partner at our behest, will request the U.S. EPA to register the same already approved use pattern in maize for use on MON 87429 maize.

U. S. EPA has registered the use of quizalofop[5] on maize up to 0.083 lb from V2 to V6 stage of development. Monsanto will request the U.S. EPA to register the same already approve use pattern in maize for use on MON 87429 maize.

Glyphosate use over the top of MON 87429 will follow the current approved use pattern of glyphosate[6]. Glyphosate is labeled for preplant applications on conventional and herbicide-tolerant maize hybrids and for in-crop postemergence applications on glyphosate-tolerant hybrids only.

I.C.3. Submissions to Foreign Government Agencies

Consistent with our commitments to the Biotechnology Industry Organization (BIO) and Excellence Through Stewardship® (ETS)[7], Monsanto will meet applicable regulatory requirements for MON 87429 in the country of intended production and for major import countries, as identified in the trade assessment process that have functioning regulatory systems to assure global compliance and support the flow of international trade. Monsanto will continue to monitor other countries that are key importers of maize from the U.S., for the development of formal biotechnology approval processes. If new functioning regulatory processes are developed, Monsanto will re-evaluate its stewardship plans and make appropriate modifications to minimize the potential for trade disruption.

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[3] Federal Register, 77 FR 59106-59113, Glufosinate Ammonium, Pesticide Tolerances

[4] Federal Register, 82 FR 9523-9529, 2,4-D; Pesticide Tolerances

[5] Federal Register, 83 FR 7111-7115, Quizalofop ethyl; Pesticide Tolerances

[6] Federal Register, 76 FR 27268-27271, Glyphosate; Pesticide Tolerances

® Excellence Through Stewardship is a registered trademark of Excellence Through Stewardship, Washington, DC.

[7] http://www.excellencethroughstewardship.org/.

II. THE BIOLOGY OF MAIZE

The biology of maize has been well documented (Anderson and de Vicente, 2010; Farnham et al., 2003; OGTR, 2008). In addition to the wealth of information on maize biology, the Organisation for Economic Co-operation and Development authored a Consensus Document (OECD, 2003) on the biology of maize. This document provides key information regarding:

- general description of maize biology, including taxonomy and morphology and use of maize as a crop plant
- agronomic practices in maize cultivation
- geographic centers of origin
- reproductive biology
- cultivated maize as a volunteer weed
- inter-species/genus introgression into relatives and interactions with other organisms
- summary of the ecology of maize

Additional information on the biology and uses of maize can also be found on the Australian Government Department of Health and Ageing (Office of the Gene Technology Regulator) web site (OGTR, 2008), and in the USDA-ARS GRIN database (USDA-ARS, 2015). The taxonomic information for maize is available in the USDA's PLANTS Profile (USDA-NRCS, 2019).

To support the evaluation of the plant pest potential of MON 87429 relative to conventional maize, additional information regarding several aspects of maize biology can be found elsewhere in this petition. This includes: agronomic practices for maize in Section VIII; volunteer management of maize in Section VIII.H; and inter-species/genus introgression potential in Section IX.D.

II.A. Maize as a Crop

Maize is widely grown in nearly all areas of the world and is the largest grain crop in the world, ahead of both wheat (*Triticum sp.*) and rice (*Oryza sativa* L.), in total metric ton production (FAOSTAT, 2019). In the 2016/2017 marketing year, world maize area was approximately 194 million hectares (ha) with a total grain production of approximately 1,122 million metric tons (MMT) (USDA-FAS, 2018). The top five production regions were: USA (385 MMT), China (264 MMT), Brazil (99 MMT), the European Union (62 MMT), and Argentina (41 MMT) (USDA-FAS, 2018). In the U.S., maize is grown in most states and in 2017, its production value of over $49 billion was the highest of any crop (USDA-NASS, 2019a).

In industrialized countries maize has two major uses: (1) as animal feed in the form of grain, forage or silage; and (2) as a raw material for wet- or dry-milled processed products such as high fructose maize syrup, oil, starch, glucose, dextrose and ethanol. By-products of the wet- and dry- mill processes are also used as animal feed. These

processed products are used as ingredients in many industrial applications and in human food products. Most maize produced in industrialized countries is used as animal feed or for industrial purposes, but maize remains an important food staple in many developing regions, especially sub-Saharan Africa and Central America, where it is frequently the mainstay of human diets (Morris, 1998).

Maize is a familiar plant that has been rigorously studied due to its use as a staple food/feed and the economic opportunity it brings to growers. Archeological and genetic evidence suggests that maize domestication began in southern Mexico approximately 9,000 years ago, and that it was derived from Balsas teosinte, *Zea mays* subsp. *parviglumis* (Kistler et al., 2018; Matsuoka et al., 2002; Piperno et al., 2009). Although grown extensively throughout the world, maize is not considered a threat to invade natural or agricultural ecosystems. Maize does not establish self-sustaining populations outside of cultivation (Crawley et al., 2001; OECD, 2003; Raybould et al., 2012). This lack of weediness may reflect its poor competitive ability (Olson and Sander, 1988), lack of seed dormancy, and barriers to seed dispersal, as maize cobs retain seed and are covered in a husk (Wilkes, 1972). A number of other characteristics common in weeds, such as rapid flowering following emergence, are lacking in maize (Keeler, 1989). Today, the majority of U.S. maize acreage is planted to hybrids, a practice that started in the 1920s (Wych, 1988). Maize hybrids have advantages in yield and plant vigor associated with heterosis, also known as hybrid vigor (Duvick, 1999).

Conventional plant breeding results in selection of desirable characteristics in a plant through the generation of unique combinations of genes obtained by intra- and inter-specific crossing, mutation breeding or utilization of other traditional breeding methodologies. However, there is a limit to the genetic diversity that is available for use and selection with conventional plant breeding. Biotechnology, as an additional tool to conventional breeding, offers access to greater genetic diversity than conventional breeding alone, resulting in expression of traits that are highly desirable to growers and downstream crop users.

II.B. Characteristics of the Recipient Plant

The MON 87429 transformation was conducted with inbred maize line LH244, a patented maize line assigned to Holden's Foundation Seeds LLC in 2001 (U.S. Patent #6,252,148). LH244 is a medium season yellow dent maize line of Stiff Stalk background that is best adapted to the central regions of the U.S. corn belt.

Following transformation of immature LH244 embryos, a single transformed plant was selected and self-pollinated to increase seed supplies. An inbred line homozygous for MON 87429 was selected and then used to produce other MON 87429 materials for product testing, safety assessment studies, and commercial hybrid development. The non-transformed LH244 was used to produce conventional maize comparators (hereafter referred to as conventional controls) in the safety assessment of MON 87429.

II.C. Maize as a Test System in Product Safety Assessment

In studies utilizing hybrid maize, the test is a hybrid of LH244 (MON 87429 expressing DMO, PAT, FT_T and CP4 EPSPS proteins) × HCL617 unless otherwise noted (Figure IV-4). LH244 × HCL617 was used as near isogenic, conventional control for this submission (hereafter referred to as conventional control). LH244 was used as the conventional control in molecular characterization studies. LH244 × HCL617 was used as the conventional control in compositional analysis and in phenotypic, agronomic and environmental interactions assessments. Where appropriate conventional commercial maize hybrids (hereafter referred to as reference hybrids) were used to establish ranges of natural variability or responses representative of commercial maize hybrids. Reference hybrids used at each field trial location were selected based on their availability and agronomic fit for the respective geographic regions.

To conduct the studies reported in this petition, appropriate MON 87429 test materials were generated for the molecular characterization (Sections III and IV), protein characterization and expression analysis (Section V), compositional analysis (Section VI), and phenotypic, agronomic and environmental interactions assessment (Section VII). The full molecular characterization studies and initiation of commercial breeding efforts were conducted with the R3 generation (Figure IV-4). Protein characterization and expression analysis, composition analysis, and phenotypic, agronomic and environmental interactions assessment were conducted with MON 87429 breeding generation R3F1.

III. DESCRIPTION OF THE GENETIC MODIFICATION

This section provides a description of the transformation process and plasmid vector used in the development of MON 87429 (Figure III-1 and Figure III-2). Molecular analyses are an integral part of the characterization of maize products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into a single locus in the plant genome. This helps ensure that only the intended DNA encoding the desired trait(s) is integrated into the plant genome and facilitates the molecular characterization of the product. Information provided here allows for the identification of the genetic material present in the transformation vector delivered to the host plant and for an analysis of the data supporting the characterization of the DNA inserted in the plant found in Section IV.

III.A. Description of Transformation Plasmid PV-ZMHT519224

Plasmid vector PV-ZMHT519224 was used in the transformation of maize to produce MON 87429 and its plasmid map is shown in Figure III-2. A detailed description of the genetic elements and their prefixes (e.g., B, P, L, I, TS, CS, T, and OR) in PVZMHT519224 is provided in Table III-1. Plasmid vector PV-ZMHT519224 is approximately 17.8 kb in length and contains a single T-DNA that is delineated by Right and Left Border regions. The T-DNA contains the *pat*, *dmo*, *ft_t*, and *cp4 epsps* expression cassettes. During transformation, the T-DNA was inserted into the maize genome. Following transformation, traditional breeding, segregation, selection and screening were used to isolate those plants that contained the *pat*, *dmo*, *ft_t*, and *cp4 epsps* expression cassettes and did not contain the backbone sequences.

The *pat* expression cassette contains the following genetic elements: promoter, 5' UTR, and intron sequences for a ubiquitin gene (*Ubq*) from *Erianthus ravennae* (plume grass), the *pat* gene from *Streptomyces viridochromogenes,* and the 3' UTR sequence of the *fructose-bisphosphate aldolase* (*Fba*) gene from *Setaria italica* (foxtail millet). The *dmo* expression cassette contains the following genetic elements: Promoter, 5' UTR, and intron sequences for a ubiquitin gene (*Ubq*) from *Coix lacryma-jobi* (adlay millet), chloroplast-targeting sequence of the *Albino and pale green 6* (*Apg6)* gene from *Arabidopsis thaliana*, the *dmo* gene from *Stenotrophomonas maltophilia,* and the 3' UTR sequence of the *OsMt* gene from *Oryza sativa* (rice). The *ft_t* expression cassette contains the following genetic elements: promoter, 5' UTR, and intron sequences for a *ubiquitin* gene (*Ubq*) from *Arundo donax* (giant reed), chloroplast-targeting sequence malate dehydrogenase (*Mdh)* gene from *Arabidopsis thaliana*, the *ft_t* gene from *Sphingobium herbicidovorans*, and the 3' UTR sequence from the gene coding for a no apical meristem (*Nam*) protein domain from *Oryza sativa* (rice). The *cp4 epsps* expression cassette contains the following genetic elements: promoter and leader sequence from the 35S RNA of cauliflower mosaic virus (CaMV), 5' UTR leader sequence from the gene coding for chlorophyll a/b-binding (*CAB*) protein of *Triticum aestivum* (wheat), intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice), chloroplast-targeting sequence of the 5-enolpyruvylshikimate-3-

phosphate synthase (*ShkG*) gene from *Arabidopsis thaliana*, the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4, and 3' UTR sequence of *Zea mays* cDNA (Genbank Accession: EU974548) that contains male tissue specific siRNA target sequence, and 3' UTR sequence of the glycine-rich RNA binding protein (*Grp3*) gene from *Oryza sativa* (rice).

The backbone region of PV-ZMHT519224 contains two origins of replication for maintenance of the plasmid vector in bacteria (*ori V, ori pBR322*), and a bacterial selectable marker gene (*aadA*).

III.B. Description of the Transformation System

MON 87429 was developed through *Agrobacterium tumefaciens* mediated transformation of immature maize embryos based on the method described by (Sidorov and Duncan, 2009) utilizing PV-ZMHT519224. Immature embryos were excised from a post-pollinated maize ear of LH244. After co-culturing the excised immature embryos with *Agrobacterium* carrying the plasmid vector, the immature embryos were placed on selection medium containing glyphosate and carbenicillin disodium salt in order to inhibit the growth of untransformed plant cells and excess *Agrobacterium*, respectively. Once transformed callus developed, the callus was placed on media conducive to shoot and root development. The rooted plants (R0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment. As demonstrated in this petition (Section VII), the use of disarmed *Agrobacterium tumefaciens* strain ABI, a designated plant pest, as the transformation vector has not imparted plant pest characteristics to MON 87429.

The R0 plants generated through the transformation process described above had already been exposed to glyphosate in the selection medium and demonstrated glyphosate tolerance. The R0 plants were self-pollinated to produce R1 seed. Subsequently, the R1 population was screened for the presence of T-DNA and absence of vector backbone sequences by construct-level PCR assay and Southern blot analysis. Only plants that were homozygous positive for T-DNA and negative for vector backbone were selected for further development and their progenies were subjected to further molecular and phenotypic assessments. As is typical of a commercial event production and selection process, hundreds of different transformation events (regenerants) were generated in the laboratory using PV-ZMHT519224. After careful selection and evaluation of these events in the laboratory, greenhouse and field, MON 87429 was selected as the lead event based on superior trait efficacy, agronomic, phenotypic, and molecular characteristics (Prado et al., 2014). Studies on MON 87429 were initiated to further characterize the genetic insertion and the expressed product, and to establish the food, feed, and environmental safety relative to conventional maize. The major steps involved in the development of MON 87429 are depicted in Figure III-1. The result of this process was the production of MON 87429 maize with the *pat, dmo, ft_t* and *cp4 epsps* expression cassettes.

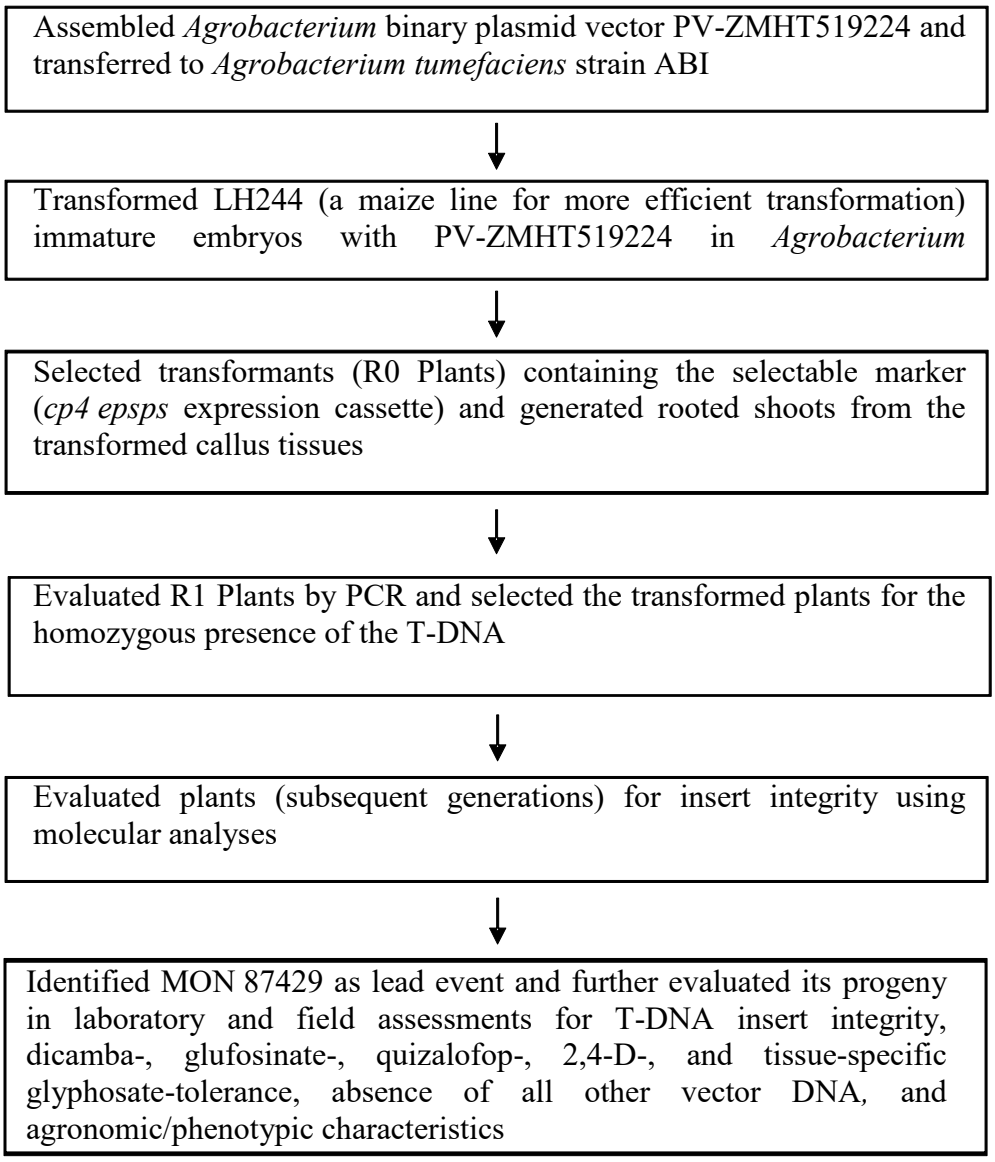


Figure III-1. Schematic of the Development of MON 87429

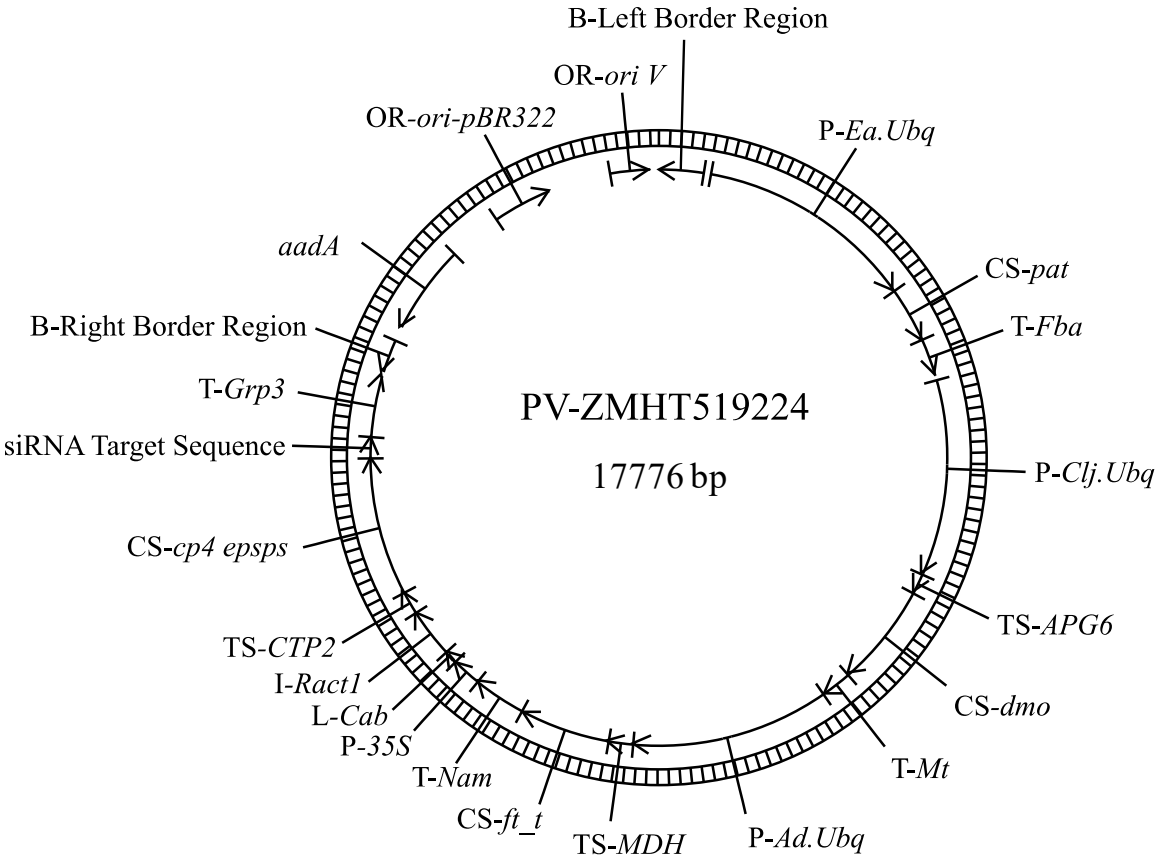


Figure III-2. Circular Map of PV-ZMHT519224

A circular map of PV-ZMHT519224 used to develop MON 87429 is shown. PV-ZMHT519224 contains one T–DNA. Genetic elements are shown on the exterior of the map.

Table III-1. Summary of Genetic Elements in PV-ZMHT519224

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T–DNA (Barker et al., 1983)
Intervening Sequence	443-513	Sequence used in DNA cloning
P[2]-*Ea.Ubq*	514-2695	Promoter, 5' UTR, and intron sequences for a ubiquitin gene (*Ubq*) from *Erianthus ravennae* (plume grass) that directs transcription in plant cells (Cornejo et al., 1993)
Intervening Sequence	2696-2700	Sequence used in DNA cloning
CS[3]-*pat*	2701-3252	Coding sequence for the phosphinothricin N–acetyltransferase (PAT) protein of *Streptomyces viridochromogenes* that confers tolerance to glufosinate (Wehrmann et al., 1996; Wohlleben et al., 1988)
T[4]-*Fba*	3253-3629	3' UTR sequence of the *fructose-bisphosphate aldolase* (*Fba*) gene from *Setaria italica* (foxtail millet) that directs polyadenylation of mRNA (Hunt, 1994)
Intervening Sequence	3630-3691	Sequence used in DNA cloning
P-*Clj.Ubq*	3692-5617	Promoter, 5' UTR, and intron sequences for a ubiquitin gene (*Ubq*) from *Coix lacryma-jobi* (adlay millet) that directs transcription in plant cells (Cornejo et al., 1993)
Intervening Sequence	5618-5627	Sequence used in DNA cloning

Table III-1. Summary of Genetic Elements in PV-ZMHT519224 (continued)

TS[5]-*APG6*	5628-5831	Codon optimized targeting sequence of the *Albino and pale green 6* (*Apg6)* gene from *Arabidopsis thaliana* encoding a chloroplast-targeted Hsp101 homologue transit peptide region that directs the protein to the chloroplast (GenBank Accession: NM_121549)
CS-*dmo*	5832-6854	Codon optimized coding sequence for the dicamba mono–oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba resistance (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	6855-6862	Sequence used in DNA cloning
T-*Mt*	6863-7162	3' UTR sequence of the *OsMt* gene from *Oryza sativa* (rice) encoding metallothionein-like protein that directs polyadenylation of mRNA (Hunt, 1994)
Intervening Sequence	7163-7170	Sequence used in DNA cloning
P-*Ad.Ubq*	7171-9127	Promoter, 5' UTR, and intron sequences for a *ubiquitin* gene (*Ubq*) from *Arundo donax* (giant reed) that directs transcription in plant cells (Cornejo et al., 1993)
Intervening Sequence	9128-9140	Sequence used in DNA cloning
TS-*MDH*	9141-9383	Targeting sequence from *Arabidopsis thaliana Mdh* gene encoding the malate dehydrogenase transit peptide region that directs the protein to the chloroplast (GenBank Accession: BT000621)
CS-*ft_t*	9384-10271	Modified version of R-2,4-dichlorophenoxypropionate dioxygenase (*Rdpa)* gene from *Sphingobium herbicidovorans* that expresses a FOPs and 2,4-D dioxygenase protein (FT_T) that confers tolerance to quizalofop and 2,4-D herbicides (Müller et al., 2006)

Table III-1. Summary of Genetic Elements in PV-ZMHT519224 (continued)

Intervening Sequence	10272-10286	Sequence used in DNA cloning
T-*Nam*	10287-10803	3' UTR sequence from the gene coding for a no apical meristem (*Nam*) protein domain containing protein from *Oryza sativa* (rice) (Hunt, 1994)
Intervening Sequence	10804-10809	Sequence used in DNA cloning
P-*35S*	10810-11133	Promoter and leader from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) that directs transcription in plant cells
Intervening Sequence	11134-11155	Sequence used in DNA cloning
L^{6}-*Cab*	11156-11216	5' UTR leader sequence from the gene coding for chlorophyll a/b-binding (*CAB*) protein of *Triticum aestivum* (wheat) that is involved in regulating gene expression (Lamppa et al., 1985)
Intervening Sequence	11217-11232	Sequence used in DNA cloning
I^{7}-*Ract1*	11233-11712	Intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 protein (McElroy et al., 1990) that is involved in regulating gene expression.
Intervening Sequence	11713-11721	Sequence used in DNA cloning
TS-*CTP2*	11722-11949	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee et al., 1987)
CS-*cp4 epsps*	11950-13317	Codon optimized coding sequence of the 5-enolpyruvylshikimate-3-phosphate synthetase (*aroA*) gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein that provides glyphosate tolerance (Barry et al., 2001; Padgette et al., 1996)
Intervening Sequence	13318-13323	Sequence used in DNA cloning
siRNA Target Sequence	13324-13524	Modified partial 3' UTR sequence of *Zea mays* cDNA (Genbank Accession: EU974548) that contains male tissue specific siRNA target sequence (Brodersen and Voinnet, 2006)

Table III-1. Summary of Genetic Elements in PV-ZMHT519224 (continued)

Intervening Sequence	13525-13532	Sequence used in DNA cloning
T-*Grp3*	13533-14143	3' UTR sequence of the glycine-rich RNA binding- protein (*Grp3*) gene from *Oryza sativa* (rice) encoding the GRP3 protein that directs polyadenylation of mRNA (Hunt, 1994)
Intervening Sequence	14144-14184	Sequence used in DNA cloning
B-Right Border Region	14185-14515	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T–DNA (Depicker et al., 1982; Zambryski et al., 1982)
Vector Backbone		
Intervening Sequence	14516-14659	Sequence used in DNA cloning
aadA	14660-15548	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3"(9) –*O*–nucleotidyltransferase from the transposon Tn*7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	15549-16082	Sequence used in DNA cloning
OR[8]-*ori-pBR322*	16083-16671	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	16672-17293	Sequence used in DNA cloning
OR-*ori V*	17294-17690	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	17691-17776	Sequence used in DNA cloning

[1] B, Border
[2] P, Promoter
[3] CS, Coding Sequence
[4] T, Transcription Termination Sequence
[5] TS, Targeting Sequence
[6] L, Leader
[7] I, Intron
[8] OR, Origin of Replication

III.C. The *pat* Coding Sequence and PAT Protein

The *pat* expression cassette contains the *pat* gene encoding a protein of 183 amino acids (Figure III-3). MON 87429 expresses a 25.5 kDa PAT protein, which consists of a single polypeptide of 182 amino acids after the removal of the lead methionine that is cleaved during a co-translational process (Wehrmann et al., 1996; Wohlleben et al., 1988). The *pat* open reading frame in the expression cassette includes sequence from *S. viridochromogenes* that encodes the PAT protein. The expression of PAT protein confers glufosinate tolerance.

```
1     MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL

51    ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL

101   GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA

151   AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI
```

Figure III-3. Deduced Amino Acid Sequence of the PAT Protein

The amino acid sequence of the MON 87429 PAT protein was deduced from the full-length coding nucleotide sequence present in PV-ZMHT519224 (See Table III-1 for more detail). The lead methionine (boxed with solid line) of the PAT protein produced in MON 87429 is cleaved during a co-translational process in MON 87429.

III.D. The *dmo* Coding Sequence and DMO Protein

The *dmo* expression cassette contains the *dmo* gene. The *dmo* expression cassette encodes a precursor protein of 408 amino acids (340 amino acids encoded by the *dmo* gene and 68 amino acids encoded by the *APG6* gene for targeting the DMO protein into chloroplasts) (Figure III-4). MON 87429 expresses two forms of mature DMO protein due to alternative processing of the chloroplast transit peptide (CTP). One form consists of 341 amino acids, which includes 340 amino acids encoded by the *dmo* gene and 1 amino acid (cysteine) encoded by the *APG6* gene. The other form of the DMO protein consists of 340 amino acids encoded by the *dmo* gene. The two forms of mature DMO protein expressed in MON 87429 are indistinguishable by Coomassie stain of SDS-PAGE and western blot analysis because the difference in molecular weight between these two forms is very small. Therefore, only a ~38.4 kDa DMO protein band is observed by Coomassie stain of SDS-PAGE and western blot analysis. The *dmo* open reading frame in the expression cassette includes a codon optimized sequence from *S. maltophilia* that encodes the DMO protein (Herman et al., 2005; Wang et al., 1997). The expression of the DMO protein confers tolerance to dicamba herbicide.

```
1    MATATTTATA AFSGVVSVGT ETRRIYSFSH LQPSAAFPAK PSSFKSLKLK
51   QSARLTRRLD HRPFVVRCML TFVRNAWYVA ALPEELSEKP LGRTILDTPL
101  ALYRQPDGVV AALLDICPHR FAPLSDGILV NGHLQCPYHG LEFDGGGQCV
151  HNPHGNGARP ASLNVRSFPV VERDALIWIW PGDPALADPG AIPDFGCRVD
201  PAYRTVGGYG HVDCNYKLLV DNLMDLGHAQ YVHRANAQTD AFDRLEREVI
251  VGDGEIQALM KIPGGTPSVL MAKFLRGANT PVDAWNDIRW NKVSAMLNFI
301  AVAPEGTPKE QSIHSRGTHI LTPETEASCH YFFGSSRNFG IDDPEMDGVL
351  RSWQAQALVK EDKVVVEAIE RRRAYVEANG IRPAMLSCDE AAVRVSREIE
401  KLEQLEAA
```

Figure III-4. Deduced Amino Acid Sequence of APG6 Chloroplast Targeting Sequence and the DMO Protein

The amino acid sequence of the MON 87429 DMO precursor protein was deduced from the full-length coding nucleotide sequence present in PV-ZMHT519224 (See Table III-1 for more detail). The first 68 amino acids of the precursor protein (underlined) are the CTP from *APG6* gene. The CTP targets MON 87429 DMO precursor protein to the chloroplast and is partially cleaved in the chloroplast producing the mature 341 amino acid and 340 amino acid DMO proteins that begin with the cysteine at position 68 and methionine at position 69, respectively. The double underline shows the cysteine amino acid from APG6.

III.E. The *ft_t* Coding Sequence and FT_T Protein

The *ft_t* expression cassette contains the *ft_t* gene. The *ft_t* expression cassette encodes a precursor protein of 376 amino acids (295 amino acids encoded by the *ft_t* gene and 81 amino acids encoded by the *MDH* gene for targeting the FT_T protein into chloroplasts) (Figure III-5). MON 87429 expresses a ~36 kDa mature FT_T protein, which consists of a single polypeptide of 296 amino acids, 295 amino acids are encoded by the *ft_t* gene and 1 amino acid (alanine) is encoded by *MDH* gene due to the processing of the chloroplast transit peptide (CTP). The *ft_t* open reading frame in the expression cassette is the modified version of R-2,4-dichlorophenoxypropionate dioxygenase (*Rdpa)* gene from *Sphingobium herbicidovorans* that encodes a FOPs and 2,4-D dioxygenase protein (FT_T) (Müller et al., 2006). The expression of FT_T protein confers tolerance to quizalofop and 2,4-D herbicides.

```
1    MATATSASLF STVSSSYSKA SSIPHSRLQS VKFNSVPSFT GLKSTSLISG
51   SDSSSLAKTL RGSVTKAQTS DKKPYGFKIN AMHAALTPLT NKYRFIDVQP
101  LTGVLGAEIT GVDLREPLDD STWNEILDAF HTYQVIYFPG QAITNEQHIA
151  FSRRFGPVDP VPILKSIEGY PEVQMIRREA NESSRFIGDD WHTDSTFLDA
201  PPAAVVMRAI EVPEYGGDTG FLSMYSAWET LSPTMQATIE GLNVVHSATK
251  VFGSLYQATN WRFSNTSVKV MDVDAGDRET VHPLVVTHPV TGRRALYCNQ
301  VYCQKIQGMT DAESKSLLQF LYEHATKFDF TCRVRWKKDQ VLVWDNLCTM
351  HRAVPDYAGK FRYLTRTTVA GDKPSR
```

Figure III-5. Deduced Amino Acid Sequence of MDH Chloroplast Targeting Sequence and the FT_T Protein

The amino acid sequence of the MON 87429 FT_T precursor protein was deduced from the full-length coding nucleotide sequence present in PV-ZMHT519224 (See Table III-1 for more detail). The first 81 amino acids of the precursor protein (underlined) are the CTP from *MDH* gene. MDH targets FT_T protein to the chloroplast and is cleaved in the chloroplast producing the mature 296 amino acid FT_T protein that begins with the alanine at position 81. The double underline shows the alanine amino acid from MDH that is the N-terminus of the mature FT_T protein.

III.F. The *cp4 epsps* Coding Sequence and CP4 EPSPS Protein

The *cp4 epsps* expression cassette contains the *cp4 epsps* gene. The *cp4 epsps* expression cassette encodes a precursor protein of 531 amino acids (455 amino acids encoded by the *cp4 epsps* gene and 76 amino acids encoded by the *CTP2* gene for targeting the CP4 EPSPS protein into chloroplasts) (Figure III-6). MON 87429 expresses a 44 kDa CP4 EPSPS protein, consisting of a single polypeptide of 455 amino acids starting at the methionine position 77 (Padgette et al., 1996) after a complete cleavage of the chloroplast transit peptide (CTP2). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS

(Barry et al., 2001; Padgette et al., 1996). The CP4 EPSPS protein is similar and functionally equivalent to endogenous plant EPSPS enzymes, but has a much-reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Barry et al., 2001; Padgette et al., 1996). The presence of this protein renders the plant tolerant to glyphosate.

```
1    MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
51   LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101  TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD
151  RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR
201  DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF
251  PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301  VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL
351  RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401  HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451  DTKAA
```

Figure III-6. Deduced Amino Acid Sequence of the CP4 EPSPS Protein

The amino acid sequence of the MON 87429 CP4 EPSPS protein was deduced from the full-length coding nucleotide sequence present in PV-ZMHT519224 (See Table III-1 for more detail). Another mature form of CP4 EPSPS protein containing 454 amino acids, resulting from the cleavage of the lead methionine (boxed with dash line) was also observed.

III.G. Regulatory Sequences

The *pat* coding sequence in MON 87429 is under the regulation of the promoter, 5' untranslated region (UTR) and intron for a ubiquitin gene (*Ubq*) from *Erianthus ravennae* (plume grass) that direct transcription in plant cells (Cornejo et al., 1993). The *pat* coding sequence also utilizes the 3′ UTR sequence of the *fructose-bisphosphate aldolase* (*Fba*) gene from *Setaria italica* (foxtail millet) that directs polyadenylation of mRNA (Hunt, 1994).

The *dmo* coding sequence in MON 87429 is under the regulation of the promoter, 5' UTR and intron for a ubiquitin gene (*Ubq*) from *Coix lacryma-jobi* (adlay millet) that direct transcription in plant cells (Cornejo et al., 1993). The *dmo* coding sequence utilizes a codon optimized targeting sequence of the *Albino and pale green 6* (*Apg6)* gene from *Arabidopsis thaliana* encoding a chloroplast-targeted Hsp101 homologue transit peptide region that directs transport of the DMO protein to the chloroplast (GenBank Accession: NM_121549). The *dmo* coding sequence also utilizes the 3' UTR sequence of the *OsMt* gene from *Oryza sativa* (rice) encoding metallothionein-like protein that directs polyadenylation of mRNA (Hunt, 1994).

The *ft_t* coding sequence in MON 87429 is under the regulation of the promoter, 5' UTR, and intron for a *ubiquitin* gene (*Ubq*) from *Arundo donax* (giant reed) that directs transcription in plant cells (Cornejo et al., 1993). The *ft_t* coding sequence utilizes a chloroplast targeting sequence from *Arabidopsis thaliana Mdh* gene encoding the malate dehydrogenase transit peptide region that directs transport of the FT_T protein to the chloroplast (GenBank Accession: BT000621). The *ft_t* coding sequence also utilizes the 3' UTR sequence from the gene coding for a no apical meristem (*Nam*) protein domain containing protein from *Oryza sativa* (rice) that directs polyadenylation of mRNA (Hunt, 1994).

The *cp4 epsps* coding sequence in MON 87429 is under the regulation of the promoter and leader from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) that direct transcription in plant cells. The *cp4 epsps* coding sequence also utilizes the 5' UTR leader sequence from the gene coding for chlorophyll a/b-binding (CAB) protein of *Triticum aestivum* (wheat) that is involved in regulating gene expression (Lamppa et al., 1985), and the intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 (*Ract1*) protein that is also involved in regulating gene expression (McElroy et al., 1990). The *cp4 epsps* coding sequence utilizes a chloroplast targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the CP4 EPSPS protein to the chloroplast (Herrmann, 1995; Klee et al., 1987). The *cp4 epsps* coding sequence is regulated by a modified partial 3' UTR sequence of *Zea mays* cDNA (Genbank Accession: EU974548) that contains a target sequence recognized by endogenous male tissue specific siRNAs (Brodersen and Voinnet, 2006), which suppresses *cp4 epsps* gene expression in maize male tissue (Yang et al., 2018). The *cp4 epsps* coding sequence also utilizes a 3' UTR sequence of the glycine-rich RNA-binding protein (*Grp3*) gene from *Oryza sativa* (rice) encoding the GRP3 protein that directs polyadenylation of mRNA (Hunt, 1994).

III.H. T-DNA Border Regions

PV-ZMHT519224 contains Left and Right Border regions (Figure III-2 and Table III-1) that were derived from *A. tumefaciens* plasmids. The border regions each contain a nick site that is the site of DNA exchange during transformation (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer of T-DNA into the maize genome. As demonstrated in this petition (Section VII), the use of genetic elements from *A. tumefaciens*, a designated plant pest, has not imparted plant pest characteristics to MON 87429.

III.I. Genetic Elements Outside the T-DNA Border Regions

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-ZMHT519224 in bacteria and are referred to as plasmid backbone. The selectable marker, *aadA* is the coding sequence for an aminoglycoside-modifying enzyme, 3"(9) –*O*–nucleotidyltransferase from the transposon Tn*7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance in *E. coli* and *Agrobacterium* for use in molecular cloning. The origin of

replication, *ori-pBR322*, is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe, 1979). The origin of replication, *ori V*, is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host range plasmid RK2 from *Agrobacterium* (Stalker et al., 1981). Because these elements are outside the T-DNA border regions, they are not expected to be transferred into the maize genome. The absence of the backbone and other unintended plasmid sequence in MON 87429 was confirmed by sequencing and bioinformatic analyses (Section IV).

IV. CHARACTERIZATION OF THE GENETIC MODIFICATION

This section describes the methods and results of a comprehensive molecular characterization of the genetic modification present in MON 87429. It provides information on the DNA insertion(s) into the plant genome of MON 87429, and additional information regarding the arrangement and stability of the introduced genetic material. The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 30, 31, 32, and 33 (Codex Alimentarius, 2009).

IV.A. Description of Methodology Used to Characterize MON 87429

A schematic representation of the next generation sequencing (NGS) methodology and the basis of the characterization using NGS and PCR sequencing are illustrated in Figure IV-1 below. Appendix B defines the test, control and reference substances, and provides an additional overview of these techniques, their use in DNA characterization in maize plants and the materials and methods.

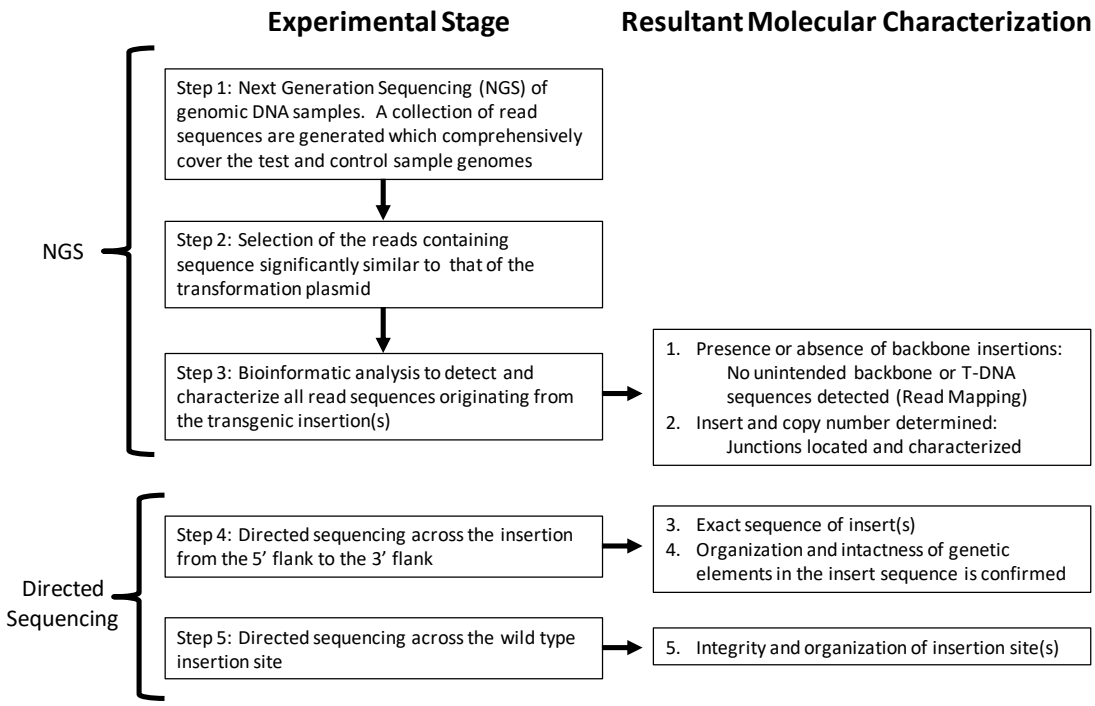


Figure IV-1. Molecular Characterization using Sequencing and Bioinformatics

Genomic DNA from MON 87429 (Test) and the conventional control was sequenced using technology that produces a set of short, randomly distributed sequence reads that comprehensively cover test and control genomes (Step 1). Utilizing these genomic sequence reads, bioinformatics searches are conducted to identify all sequence reads that are significantly similar to the transformation plasmid (Step 2). These identified captured reads are then mapped and analyzed to determine the presence/absence of transformation plasmid backbone sequences, identify insert junctions, and to determine the insert and copy number (Step 3). Using directed sequencing, overlapping PCR products are also produced which span any insert and the wild type insertion locus (Step 4 and Step 5 respectively); these overlapping PCR products are sequenced to allow for detailed characterization of the inserted DNA and insertion site.

The NGS method was used to characterize the genomic DNA from MON 87429 and the conventional control by generating short (~150 bp) randomly distributed sequence fragments (sequencing reads) generated in sufficient number to ensure comprehensive coverage of the sample genomes. It has been previously demonstrated that whole genome sequencing at 75× depth of coverage is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic et al., 2012). To confirm sufficient sequence coverage of the genome, the 150 bp sequence reads are analyzed to determine the coverage of a known single-copy endogenous maize gene. This establishes the depth of coverage (the median number of times each base of the genome is independently sequenced). The level of sensitivity of this method was demonstrated by detection of a positive control plasmid DNA spiked at 1 and 1/10th copy-per-genome equivalent. This confirms the method's ability to detect any sequences derived from the transformation plasmid. Bioinformatics analysis was then used to select sequencing reads that contained sequences similar to the transformation plasmid, and these were analyzed in depth to determine the number of DNA inserts. NGS was run on five breeding generations of MON 87429 and the appropriate conventional controls. Results of NGS are shown in Sections IV.B and IV.E.

The DNA inserts of MON 87429 were characterized by mapping of sequencing reads to the transformation plasmid and identifying junctions and unpaired read mappings adjacent to the junctions. Examples of five types of NGS reads are shown in Figure IV-2. The junctions of the DNA insert and the flanking DNA are unique for each insertion (Kovalic et al., 2012). Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions.

Directed sequencing (locus-specific PCR and DNA sequencing analyses, Figure IV-1, Step 4) complements the NGS method. It assesses the sequence identity of the insert relative to the corresponding sequence from the T-DNA in PV-ZMHT519224, and demonstrates that each genetic element in the insert was intact without rearrangement. It also characterizes the flank sequence beyond the insert corresponding to the genomic DNA of MON 87429. Directed sequencing results are described in Sections IV.B, IV.C and IV.D; methods are presented in Appendix B.

The stability of the T-DNA present in MON 87429 across multiple breeding generations was evaluated by NGS as described above by determining the number and identity of the DNA inserts in each generation. For a single copy T-DNA insert, two junction sequence classes are expected. In the case of an event where a single insertion locus is stably inherited over multiple breeding generations, two identical junction sequence classes would be detected in all the breeding generations tested. Results are described in Section IV.E; methods are presented in Appendix B.

Segregation analysis of the T-DNA was conducted to determine the inheritance and generational stability of the insert in maize. Segregation analysis corroborates the insert stability demonstrated by NGS and independently establishes the genetic behavior of the T-DNA. Results are described in Section IV.F.

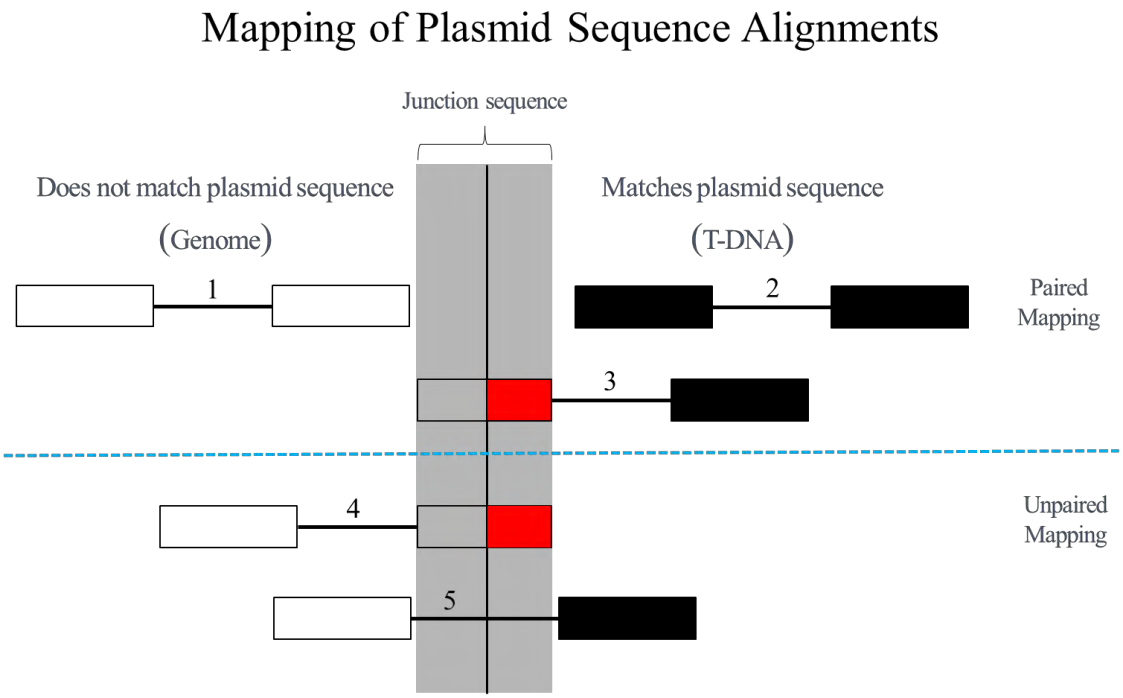


Figure IV-2. Five Types of NGS Reads

NGS yields data in the form of read pairs where sequence from each end of a size selected DNA fragment is returned. Depicted above are five types of sequencing reads/read pairs generated by NGS sequencing which can be found spanning or outside of junction points. Sequence boxes are filled red or black if it matches with plasmid sequence, and empty if it matches with genomic sequence. Grey highlighting indicates sequence reads spanning the junction. Junctions are detected by examining the NGS data for reads having portions of plasmid sequences that span less than the full read, as well as reads mapping adjacent to the junction points where their mate pair does not map to the plasmid sequence. The five types of sequencing reads/read pairs being (1) Paired and unpaired reads mapping to genomic sequence outside of the insert, greater than 99.999% of collected reads fall into this category and are not evaluated in this analysis, (2) Paired reads mapping entirely to the transformation plasmid sequence, such reads reveal the presence of transformation related sequence *in planta*, (3) Paired reads where one read maps entirely within the inserted DNA and the other read maps partially to the insert (indicating a junction point), (4) Single read mapping partially to the transformation plasmid DNA sequence (indicating a junction point) where its mate maps entirely to the genomic flanking sequence and (5) Single read mapping entirely to the transformation plasmid DNA sequence where its mate maps entirely to genomic flanking sequence, such reads are part of the junction signature.

IV.B. Characterization of the DNA Insert in MON 87429

The number of inserted DNA sequences from PV-ZMHT519224 in MON 87429 was assessed by generating a comprehensive collection of reads via NGS of MON 87429 genomic DNA using the R3 generation. A plasmid map of PV-ZMHT519224 is shown in Figure III-2. Table IV-1 provides descriptions of the genetic elements present in MON 87429. A schematic representation of the insert and flanking sequences in MON 87429 is shown in Figure IV-3. For full details on materials and methods see Appendix B.

IV.B.1. Next Generation Sequencing for MON 87429 and Conventional Control Genomic DNA

Genomic DNA from five breeding generations of MON 87429 (Figure IV-4) and conventional controls were isolated from seed and prepared for sequencing. For material and method details see Appendix B. These genomic DNA libraries were used to generate short (~150 bp) randomly distributed sequencing reads of the maize genome (Figure IV-1, Step 1).

To demonstrate sufficient sequence coverage the 150 bp sequence reads were analyzed by mapping all reads to a known single copy endogenous gene (*Zea mays* pyruvate decarboxylase (*pdc3*), GenBank Accession: AF370006.2) in each of the five breeding generations. The analysis of sequence coverage plots showed that the depth of coverage (i.e., the median number of times any base of the genome is expected to be independently sequenced) was 86× or greater for the five generations of MON 87429 (R3, R3F1, R4, R4F1, and R5) and the conventional control (Appendix B, Table B-1). It has been previously demonstrated that whole genome sequencing at 75× depth of coverage provides comprehensive coverage and ensures detection of inserted DNA (Kovalic et al., 2012).

To demonstrate the method's ability to detect any sequences derived from the PV-ZMHT519224 transformation plasmid, a sample of conventional control genomic DNA spiked with PV-ZMHT519224 DNA was analyzed by NGS and bioinformatics. The level of sensitivity of this method was demonstrated to a level of one genome equivalent and 1/10th genome equivalent, 100% nucleotide identity was observed over 100% of PV-ZMHT519224 (Appendix B, Table B-2). This result demonstrates that all nucleotides of PV-ZMHT519224 are observed by the sequencing and bioinformatic assessments performed and that a detection level of at least 1/10th genome equivalent was achieved for the plasmid DNA sequence assessment.

Table IV-1. Summary of Genetic Elements in MON 87429

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
5' Flanking DNA	1-1029	DNA sequence flanking the 5' end of the insert
B[3]-Left Border Region[r1]	1030-1288	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T–DNA (Barker et al., 1983)
Intervening Sequence	1289-1359	Sequence used in DNA cloning
P[4]-*Ea.Ubq*	1360-3541	Promoter, 5' UTR, and intron sequences for a ubiquitin gene (*Ubq*) from *Erianthus ravennae* (plume grass) that directs transcription in plant cells (Cornejo et al., 1993)
Intervening Sequence	3542-3546	Sequence used in DNA cloning
CS[5]-*pat*	3547-4098	Coding sequence for the phosphinothricin N–acetyltransferase (PAT) protein of *Streptomyces viridochromogenes* that confers tolerance to glufosinate (Wehrmann et al., 1996; Wohlleben et al., 1988)
T[6]-*Fba*	4099-4475	3' UTR sequence of the *fructose-bisphosphate aldolase* (*Fba*) gene from *Setaria italica* (foxtail millet) that directs polyadenylation of mRNA (Hunt, 1994)
Intervening Sequence	4476-4537	Sequence used in DNA cloning
P-*Clj.Ubq*	4538-6463	Promoter, 5' UTR, and intron sequences for a ubiquitin gene (*Ubq*) from *Coix lacryma-jobi* (adlay millet) that directs transcription in plant cells (Cornejo et al., 1993)
Intervening Sequence	6464-6473	Sequence used in DNA cloning
TS[7]-*APG6*	6474-6677	Codon optimized targeting sequence of the *Albino and pale green 6* (*Apg6)* gene from *Arabidopsis thaliana* encoding a chloroplast-targeted Hsp101 homologue transit peptide region that directs the protein to the chloroplast (GenBank Accession: NM 121549)

Table IV-1. Summary of Genetic Elements in MON 87429 (continued)

CS-*dmo*	6678-7700	Codon optimized coding sequence for the dicamba mono–oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba resistance (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	7701-7708	Sequence used in DNA cloning
T-*Mt*	7709-8008	3' UTR sequence of the *OsMt* gene from *Oryza sativa* (rice) encoding metallothionein-like protein that directs polyadenylation of mRNA (Hunt, 1994)
Intervening Sequence	8009-8016	Sequence used in DNA cloning
P-*Ad.Ubq*	8017-9973	Promoter, 5' UTR, and intron sequences for a *ubiquitin* gene (*Ubq*) from *Arundo donax* (giant reed) that directs transcription in plant cells (Cornejo et al., 1993)
Intervening Sequence	9974-9986	Sequence used in DNA cloning
TS-*MDH*	9987-10229	Targeting sequence from *Arabidopsis thaliana Mdh* gene encoding the malate dehydrogenase transit peptide region that directs the protein to the chloroplast (GenBank Accession: BT000621)
CS-*ft_t*	10230-11117	Modified version of R-2,4-dichlorophenoxypropionate dioxygenase (*Rdpa)* gene from *Sphingobium herbicidovorans* that expresses a FOPs and 2,4-D dioxygenase protein (FT_T) that confers tolerance to quizalofop and 2,4-D herbicides (Müller et al., 2006)
Intervening Sequence	11118-11132	Sequence used in DNA cloning
T-*Nam*	11133-11649	3' UTR sequence from the gene coding for a no apical meristem (*Nam*) protein domain containing protein from *Oryza sativa* (rice) (Hunt, 1994)
Intervening Sequence	11650-11655	Sequence used in DNA cloning

Table IV-1. Summary of Genetic Elements in MON 87429 (continued)

P-*35S*	11656-11979	Promoter and leader from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) that directs transcription in plant cells
Intervening Sequence	11980-12001	Sequence used in DNA cloning
L[8]-*Cab*	12002-12062	5' UTR leader sequence from the gene coding for chlorophyll a/b-binding (*CAB*) protein of *Triticum aestivum* (wheat) that is involved in regulating gene expression (Lamppa et al., 1985)
Intervening Sequence	12063-12078	Sequence used in DNA cloning
I[9]-*Ract1*	12079-12558	Intron and flanking UTR sequence of the *act1* gene from *Oryza sativa* (rice) encoding rice Actin 1 protein (McElroy et al., 1990) that is involved in regulating gene expression.
Intervening Sequence	12559-12567	Sequence used in DNA cloning
TS-*CTP2*	12568-12795	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee et al., 1987)
CS-*cp4 epsps*	12796-14163	Codon optimized coding sequence of the 5-enolpyruvylshikimate-3-phosphate synthetase (*aroA*) gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein that provides glyphosate tolerance (Barry et al., 2001; Padgette et al., 1996)
Intervening Sequence	14164-14169	Sequence used in DNA cloning
siRNA Target Sequence	14170-14370	Modified partial 3' UTR sequence of *Zea mays* cDNA (Genbank Accession: EU974548) that contains male tissue specific siRNA Target Sequence (Brodersen and Voinnet, 2006)
Intervening Sequence	14371-14378	Sequence used in DNA cloning
T-*Grp3*	14379-14989	3' UTR sequence of the glycine-rich RNA binding- protein (*Grp3*) gene from *Oryza sativa* (rice) encoding the GRP3 protein that directs polyadenylation of mRNA (Hunt, 1994)

Table IV-1. Summary of Genetic Elements in MON 87429 (continued)

Intervening Sequence	14990-15030	Sequence used in DNA cloning
B-Right Border Region[r1]	15031-15037	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T–DNA (Depicker et al., 1982; Zambryski et al., 1982)
3' Flanking DNA	15038-16068	Flanking DNA

[1] Although flanking sequences and intervening sequences are not functional genetic elements, they comprise a portion of the sequence.
[2] Numbering refers to the sequence of the insert in MON 87429 and adjacent DNA
[3] B, Border
[4] P, Promoter
[5] CS, Coding Sequence
[6] T, Transcription Termination Sequence
[7] TS, Targeting Sequence
[8] L, Leader
[9] I, Intron
[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87429 was truncated compared to the sequences in PV-ZMHT519224

CBI Deleted

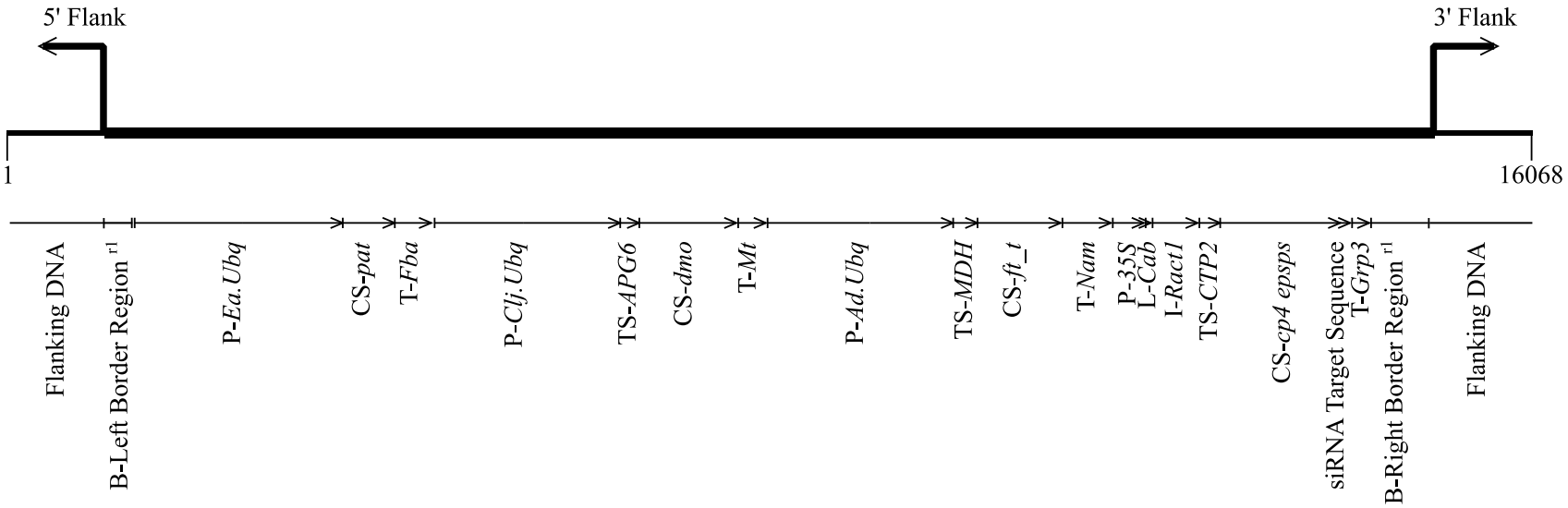


Figure IV-3. Schematic Representation of the Insert and Flanking Sequences in MON 87429

DNA derived from T-DNA of PV-ZMHT519224 integrated in MON 87429. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking sequence. Identified on the map are genetic elements within the insert. This schematic diagram may not be drawn to scale.

r1 Superscript in Left and Right Border Regions indicate that the sequence in MON 87429 was truncated compared to the sequences in PV-ZMHT519224.

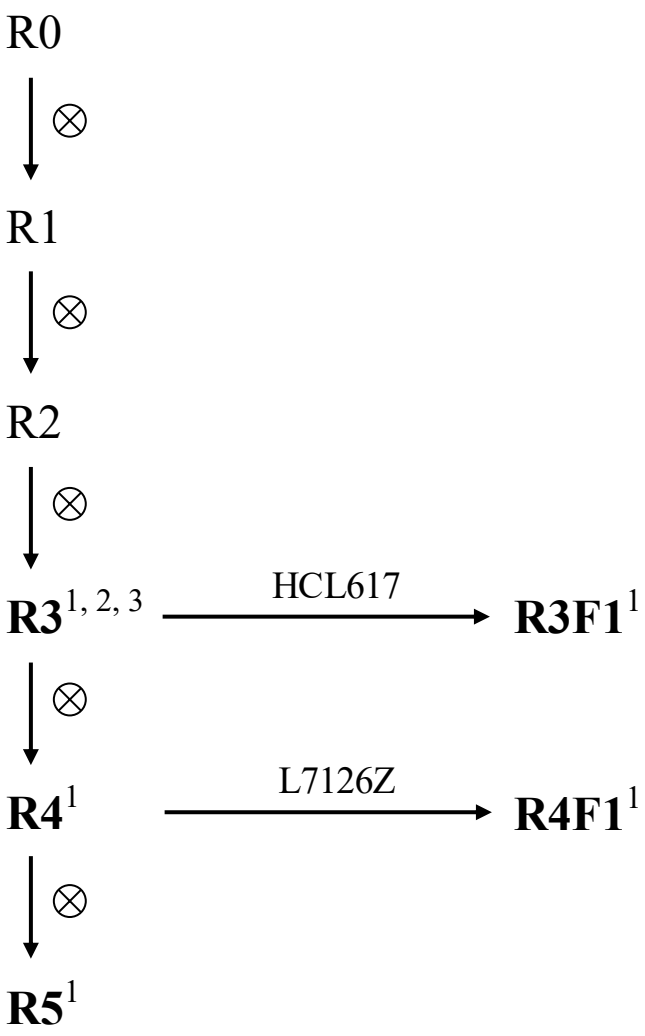


Figure IV-4. Breeding History of MON 87429

The generations used for molecular characterization and insert stability analyses are indicated in bold text. R0 (LH244) corresponds to the transformed plant, ⊗ designates self-pollination.

[1]Generations used to confirm insert stability

[2] Generation used for molecular characterization

[3] Generation used for commercial development of MON 87429

IV.B.2. Selection of Sequence Reads Containing Sequence of the PV-ZMHT519224

The transformation plasmid, PV-ZMHT519224 was transformed into the parental variety LH244 to produce MON 87429. Consequently, any DNA inserted into MON 87429 will consist of sequences that are similar to the PV-ZMHT519224 DNA sequence. Therefore, to fully characterize the DNA from PV-ZMHT519224 inserted in MON 87429, it is sufficient to analyze only the sequence reads that have significant similarity to PV-ZMHT519224 (Figure IV-1, Step 2).

Using established criteria (described in the materials and methods, Appendix B), sequence reads similar to PV-ZMHT519224 were selected from MON 87429 sequence datasets and were then used as input data for bioinformatic junction sequence analysis. PV-ZMHT519224 sequences were also compared against the conventional control sequence dataset.

IV.B.3. Determination of T-DNA Copy Number and Presence or Absence of Plasmid Vector Backbone

By mapping sequence reads to the transformation plasmid sequence and identifying junction signatures, the presence or absence of backbone sequence and the number of T-DNA insertions can be determined. For a single copy T-DNA insert sequence at a single genomic locus, a single junction signature pair and few if any reads aligning with the transformation plasmid backbone sequences are expected.

When reads from conventional maize LH244 were aligned with the transformation plasmid sequence, a number of reads mapped to the T-DNA element siRNA Target Sequence (Figure IV-5, see Panels 1 and 2; Panel 3 illustrates the read depth). The sequence alignments were expected since the siRNA Target Sequence in the T-DNA is derived from endogenous maize genomic sequence. Notably, the sequence alignment is isolated to that element alone and does not cross the element boundaries. No other sequence reads from LH244 conventional maize mapped to the transformation plasmid. Additional conventional controls (LH244 × HCL617 was the control for R3F1, and LH244 × L7126Z was the control for R4F1) evaluated in the generational stability analysis (see Table IV-3) produced comparable read maps.

When reads from the MON 87429 (R3) dataset were aligned with the transformation plasmid sequence, large numbers of reads mapped to T-DNA, and no reads were identified which aligned to the transformation plasmid backbone (Figure IV-6).

The mapping of a large number of sequencing reads from the MON 87429 (R3) dataset to the T-DNA was expected and fully consistent with the presence of the inserted DNA MON 87429. Since these sequences are identical, the mapped read mate pairs were used to distinguish their true mapping location. Also, no reads in the MON 87429 (R3) generation dataset were identified that align with the plasmid backbone. As a result, it is concluded that MON 87429 (R3) does not contain inserted sequence from the transformation plasmid backbone.

To determine the insert number in MON 87429 (R3), selected reads mapping to T-DNA as described above were analyzed to identify junctions. This bioinformatic analysis is used to find and classify partially matched reads characteristic of the ends of insertions. The number of unique junctions determined by this analysis are shown in Table IV-2.

Table IV-2. Unique Junction Sequence Class Results

Sample	Junctions Detected
MON 87429 (R3)	2
LH244	0
LH244 × HCL617	0
LH244 × L7126Z	0

Detailed mapping information of the junction sequences is shown in Figure IV-6. The location and orientation of the junction sequences relative to the T-DNA insert determined for MON 87429 are illustrated in Figure IV-6, panels 1 and 2. As shown in the figure, there are two junctions identified in MON 87429. Both junctions contain the T-DNA border sequence joined to flanking genomic sequence, indicating that they represent the sequences at the junctions of the intended T-DNA insert and the maize genome. As described earlier, no junctions were detected in any of the conventional maize control samples.

Considered together, the absence of plasmid backbone and the presence of two junctions (joining T-DNA borders and flanking sequences) indicate a single intended T-DNA at a single locus in the genome of MON 87429. Both of these junctions originate from the same locus of the MON 87429 genome and are linked by contiguous, known and expected DNA sequence. This is demonstrated by complete coverage of the sequenced reads spanning the interval between the junctions and the directed sequencing of overlapping PCR products described in Section IV.C.

Based on the comprehensive NGS and junction identification it is concluded that MON 87429 contains one copy of the T-DNA inserted into a single locus. This conclusion is confirmed by the sequencing and analysis of overlapping PCR products from this locus as described below.

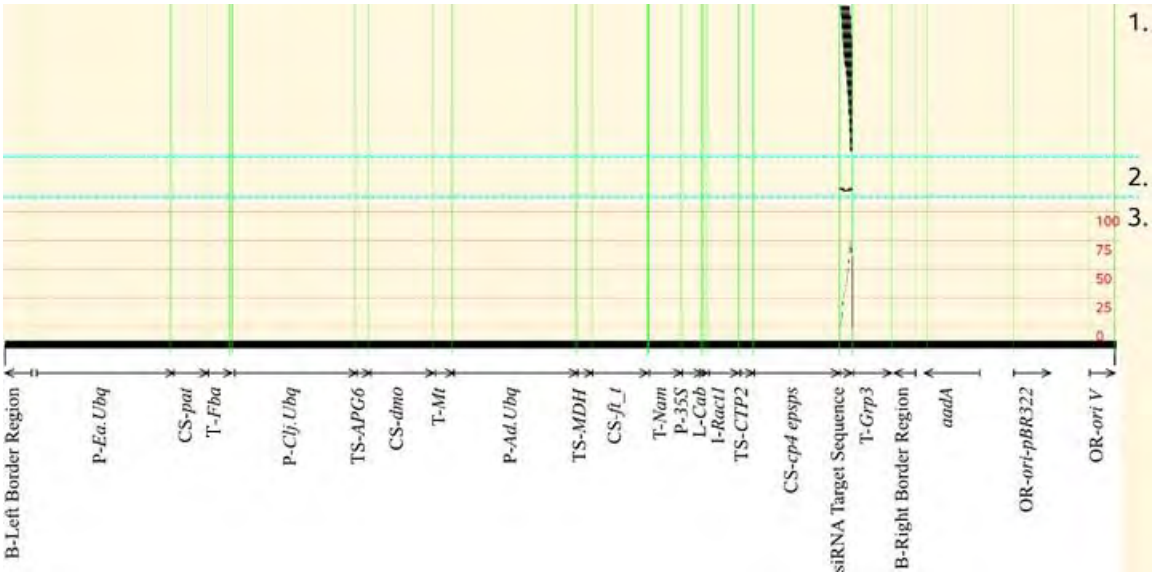


Figure IV-5. Read Mapping of Conventional Maize LH244 Versus PV-ZMHT519224

Panel 1 shows the location of unpaired mapped reads, Panel 2 shows paired mapped reads, and Panel 3 shows a representation of combined read depth for unpaired and paired reads. Vertical lines, in green, show genetic element boundaries. Comparable results were observed when read mapping LH244 × HCL617 versus PV-ZMHT519224, or read mapping LH244 × L7126Z versus PV-ZMHT519224. These additional conventional controls were used for the generational stability analysis (see Table IV-3).

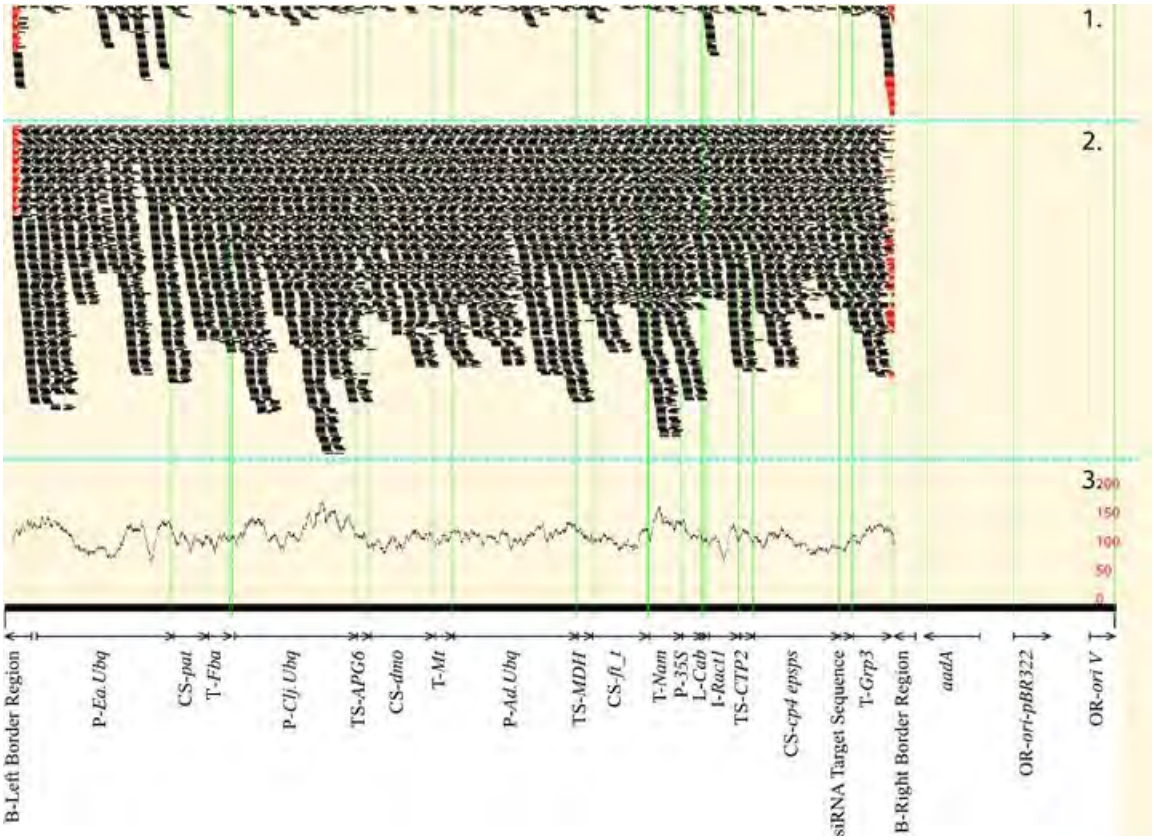


Figure IV-6. Read Mapping of MON 87429 (R3) Versus PV-ZMHT519224

Panel 1 shows the location of unpaired mapped reads. Panel 2 shows paired mapped reads and Panel 3 shows a representation of combined read depth for unpaired and paired reads. Vertical lines, in green, show genetic element boundaries. The region of flank junction sequences that aligns with transformation plasmid is shown in red. Comparable results were observed when read mapping the R3F1, R4, R4F1, and R5 generations of MON 87429 versus PV-ZMHT519224 (see Table IV-3 for the generational stability analysis).

IV.C. Organization and Sequence of the Insert and Adjacent DNA in MON 87429

The organization of the elements within the DNA insert and the adjacent genomic DNA was assessed using directed DNA sequence analysis (refer to Figure IV-1, Step 4). PCR primers were designed to amplify two overlapping regions of the MON 87429 genomic DNA that span the entire length of the insert and the adjacent DNA flanking the insert (Figure IV-7). The amplified PCR products were subjected to DNA sequencing analyses. The results of this analysis confirm that the MON 87429 insert is 14,008 bp and that each genetic element within the T-DNA is intact compared to PV-ZMHT519224. The border regions both contain small terminal deletions with the remainder of the inserted border regions being identical to the sequence in PV-ZMHT519224. The sequence and organization of the insert was also shown to be identical to the corresponding T-DNA of PV-ZMHT519224 as intended. This analysis also shows that only T-DNA elements (described in Table IV-1) were present within the inserted DNA. In addition, 1,029 base pairs flanking the 5′ end of the MON 87429 insert (Table IV-1, bases 1-1029) and 1031 base pairs flanking the 3′ end of the MON 87429 insert (Table IV-1, bases 15038-16068) were determined.

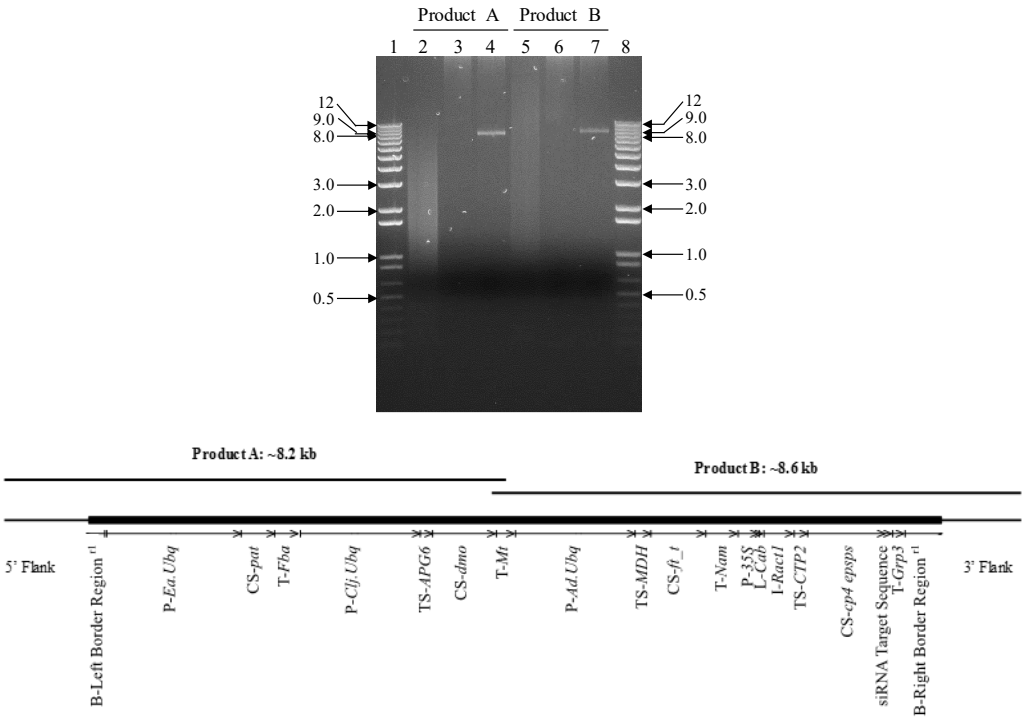


Figure IV-7. Overlapping PCR Analysis across the Insert in MON 87429

PCR was performed on both conventional control genomic DNA and genomic DNA of the R3 generation of MON 87429 using two pairs of primers to generate overlapping PCR fragments from MON 87429 for sequencing analysis. To verify size and specificity of the PCR products, 12 µl of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87429 that appears at the bottom of the figure. This figure is a representative of the data generated in the study. Lane designations are as follows:

Lane	
1	1 Kb Plus DNA Ladder
2	No template control
3	LH244 Conventional Control
4	MON 87429
5	No template control
6	LH244 Conventional Control
7	MON 87429
8	1 Kb Plus DNA Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb Plus DNA Ladder (Invitrogen) on the ethidium bromide stained gel.
[r1] Superscript in Left and Right Border Regions indicate that the sequence in MON 87429 was truncated compared to the sequences in PV-ZMHT519224

IV.D. Sequencing of the MON 87429 Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from the conventional control to examine the insertion site in conventional maize (see Figure IV-1, Step 5). The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5' end of the MON 87429 insert paired with a second primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure IV-8). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5' and 3' flanking sequences of MON 87429 indicates that 54 bases of maize genomic DNA were deleted during integration of the T-DNA. There also was a 29 base insertion in the MON 87429 5′ flanking sequence and a 31 base insertion in the MON 87429 3' flanking sequence. Such changes are common during plant transformation (Anderson et al., 2016) and these changes presumably resulted from DNA repair mechanisms in the plant during *Agrobacterium*-mediated transformation processes (Salomon and Puchta, 1998).

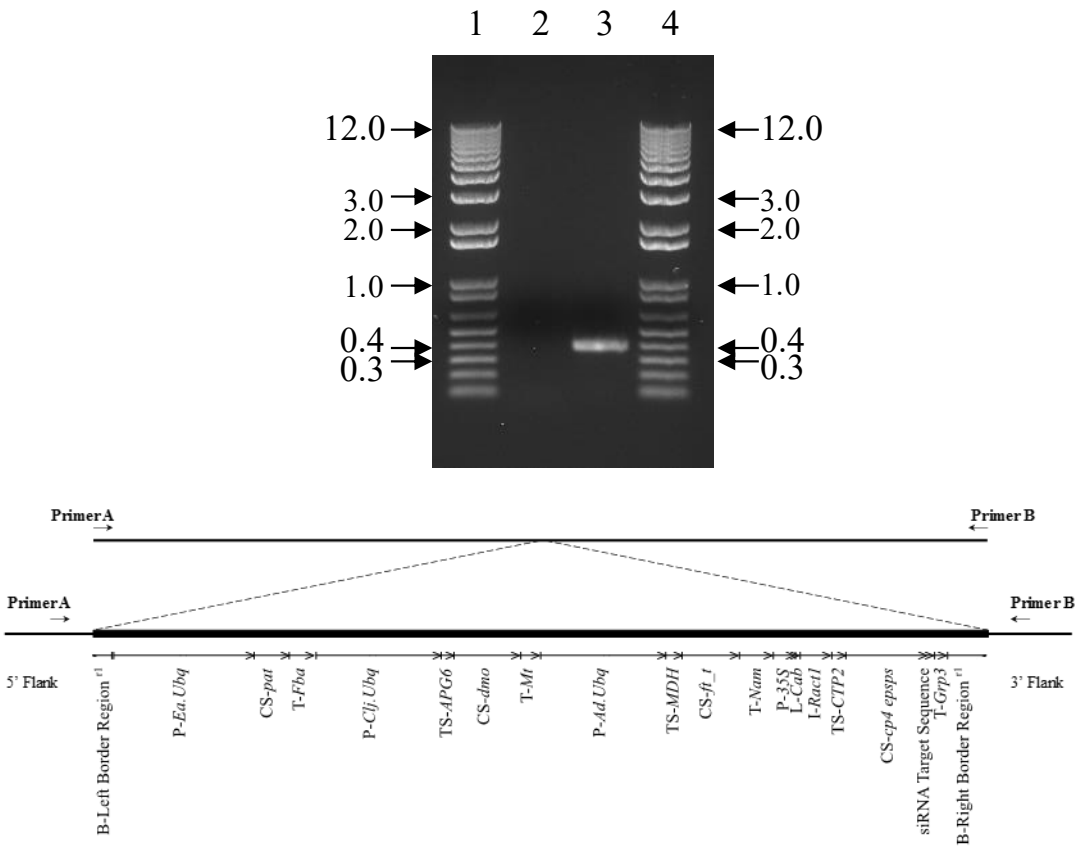


Figure IV-8. PCR Amplification of the MON 87429 Insertion Site

PCR analysis was performed to evaluate the insertion site. PCR was performed on conventional control genomic DNA using Primer A, specific to the 5′ flanking sequence, and Primer B, specific to the 3′ flanking sequence of the insert in MON 87429. The DNA generated from the conventional control PCR was used for sequencing analysis. This illustration depicts the MON 87429 insertion site in the conventional control (upper panel) and the MON 87429 insert (lower panel). Approximately 5 µl of each of the PCR reactions was loaded on the gel. This figure is representative of the data generated in the study. Lane designations are as follows:

Lane	
1	1 Kb Plus DNA Ladder
2	No template control
3	LH244 Conventional Control
4	1 Kb Plus DNA Ladder

Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb Plus DNA Ladder (Invitrogen) on the ethidium bromide stained gel.

r1 Superscript in Left and Right Border Regions indicate that the sequence in MON 87429 was truncated compared to the sequences in PV-ZMHT519224.

IV.E. Determination of Insert Stability over Multiple Generations of MON 87429

In order to demonstrate the genetic stability of the T-DNA present in MON 87429 through multiple breeding generations, NGS reads from five breeding generations of MON 87429 were mapped to the transformation plasmid for junction identification. The breeding history of MON 87429 is presented in Figure IV-4, and the specific generations tested are indicated in the figure legend. The MON 87429 (R3) generation was used for the molecular characterization analyses discussed in Sections IV.B-IV.D and shown in Figure IV-4. To assess stability, four additional generations were evaluated by NGS as previously described in Section IV.B, and compared to the fully characterized MON 87429 R3 generation. The conventional controls used for the generational stability analysis included LH244, LH244 × HCL617 and LH244 × L7126Z which represent similar background genetics to each of the analyzed MON 87429 breeding generations. Genomic DNA isolated from each of the selected generations of MON 87429 and conventional controls were used for mapping and subsequent junction identification (Table IV-3).

To determine the insert number in the MON 87429 samples, the sequences generated and selected as described above in Section IV.B.2 were analyzed to identify junctions. The number of any resultant unique junctions containing the PV-ZMHT519224 DNA sequence determined by this analysis is shown in the table below.

Table IV-3. Junction Sequence Classes Detected

Sample	Junction Sequences Detected
MON 87429 (R3)	2
MON 87429 (R3F1)	2
MON 87429 (R4)	2
MON 87429 (R4F1)	2
MON 87429 (R5)	2
LH244	0
LH244 × HCL617	0
LH244 × L7126Z	0

As shown by alignment to the full flank/insert sequence obtained from directed sequencing, a single conserved pair of junctions linked by contiguous known and expected DNA sequence is present in MON 87429 (R3). Two identical junctions are found in each of the breeding generations (R3, R3F1, R4, R4F1, and R5), confirming the insertion of a single copy of PV-ZMHT519224 T-DNA at a single locus in the genome of MON 87429, and the consistency of these junctions in the mapping data across all generations tested demonstrates that this single locus is stably maintained throughout the MON 87429 breeding process.

These results demonstrate that the single locus of integration characterized in the R3 generation of MON 87429 is found in five breeding generations of MON 87429,

confirming the stability of the insert. This comprehensive NGS and bioinformatic analysis of NGS data from multiple generations supports the conclusion that MON 87429 contains a single, stable, inserted T-DNA.

IV.F. Inheritance of the Genetic Insert in MON 87429

The MON 87429 T-DNA resides at a single locus within the maize genome and therefore should be inherited according to Mendelian principles of inheritance. During development of lines containing MON 87429, phenotypic and genotypic segregation data were recorded to assess the inheritance and stability of the MON 87429 T-DNA using Chi square (χ^2) analysis over several generations. The χ^2 analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 87429 breeding path for generating segregation data is described in Figure IV-9. The transformed R0 plant was self-pollinated to generate R1 seed. An individual plant homozygous for the MON 87429 T-DNA was identified in the R1 segregating population via a Real-Time TaqMan® PCR assay.

The homozygous positive R1 plant was self-pollinated to give rise to R2 seed. The R2 plants were self-pollinated to produce R3 seed. R3 plants homozygous for the MON 87429 T-DNA were crossed via traditional breeding techniques to a Monsanto proprietary elite inbred parent that did not contain the *dmo*, *pat*, *ft_t*, or *cp4 epsps* coding sequences to produce hemizygous R3F1 seed. The R3F1 plants were crossed again with a Monsanto proprietary elite inbred parent to produce BC1 seed. The BC1 generation was tested for the presence of the MON 87429 T-DNA by Real-Time TaqMan® PCR assay to select for hemizygous MON 87429 plants. At the BC1 generation, the MON 87429 T-DNA was predicted to segregate at a 1:1 ratio (hemizygous positive: homozygous negative) according to Mendelian inheritance principles.

Selection of hemizygous BC1 plants, followed by crossing with the Monsanto proprietary elite inbred parent, followed by testing for the presence of the T-DNA was repeated for two additional generations, to produce hemizygous BC2 seed and hemizygous BC3 seed, each at a predicted 1:1 (hemizygous positive: homozygous negative) segregation ratio according to Mendelian inheritance principles.

A Pearson's chi-square (χ^2) analysis was used to compare the observed segregation ratios of the MON 87429 T-DNA to the expected ratios. The χ^2 analysis was performed using the statistical program R Version 3.3.1 (2016-06-21).

The Chi-square was calculated as:

$$\chi^2 = \sum ((\,|\, o - e \,|\,)^2 / e)$$

where o = observed frequency of the genotype or phenotype and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$).

The results of the χ^2 analysis of the segregating progeny of MON 87429 are presented in Table IV-4. The χ^2 value in the BC1, BC2, and BC3 generations indicated no statistically significant difference between the observed and expected segregation ratios of MON 87429 T-DNA. These results support the conclusion that the MON 87429 T-DNA resides at a single locus within the maize genome and is inherited according to Mendelian principles. These results are also consistent with the molecular characterization data indicating that MON 87429 contains a single intact copy of the T-DNA inserted at a single locus in the maize genome (Section IV.B-IV.E).

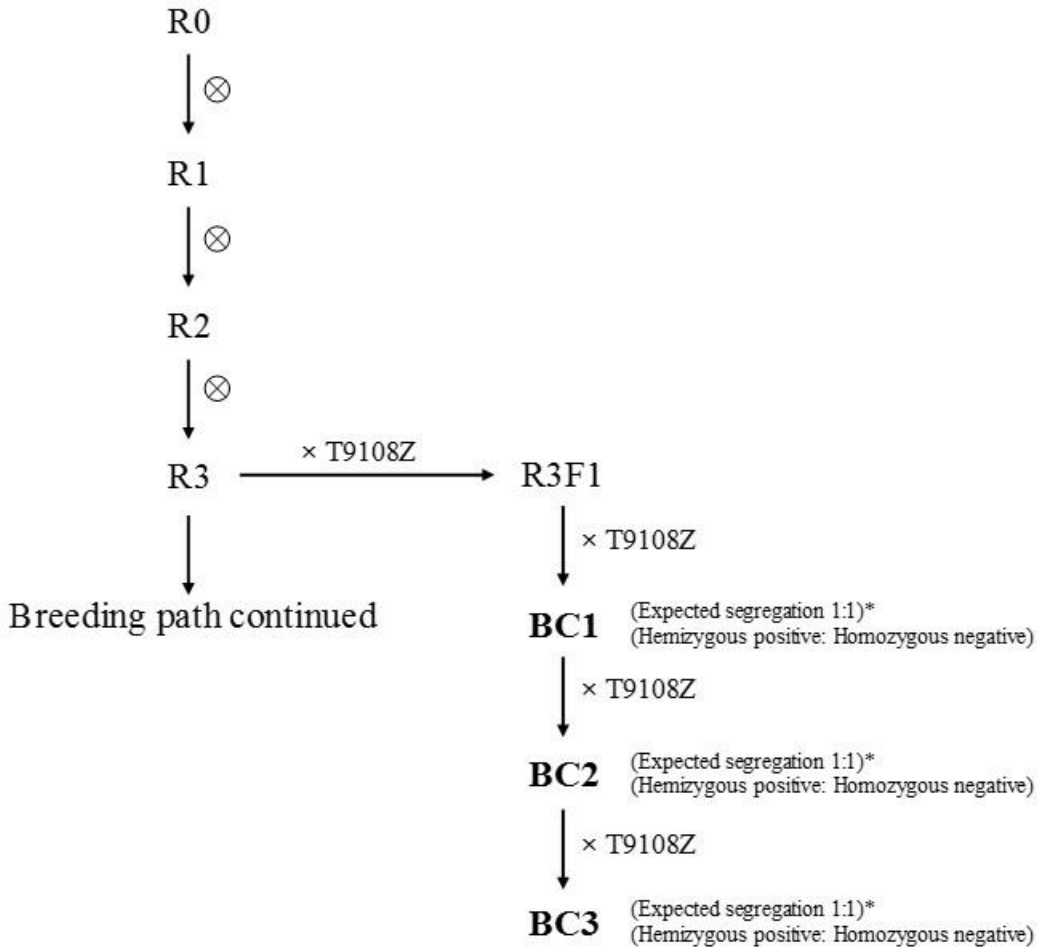


Figure IV-9. Breeding Path for Generating Segregation Data for MON 87429

*Chi-square analysis was conducted on segregation data from BC1, BC2, and BC3 generations (bolded text).
⊗: Self-Pollinated
BC: Back Cross

Table IV-4. Segregation of the Expression Cassette During the Development of MON 87429

				1:1 Segregation			
Generation	**Total Plants**	**Observed # Plant Positive**	**Observed # Plant Negative**	**Expected # Plant Positive**	**Expected # Plant Negative**	**χ 2**	**Probability**
BC1	309	148	161	154.50	154.50	0.55	0.460
BC2	236	112	124	118.00	118.00	0.61	0.435
BC3	216	97	119	108.00	108.00	2.24	0.134

IV.G. MON 87429 RHS Mechanism of Action

MON 87429 maize produces the 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) to provide maize lines with tissue-specific glyphosate tolerance to facilitate the production of hybrid maize seed. MON 87429 maize utilizes an endogenous maize regulatory element to target CP4 EPSPS mRNA for degradation in tassel tissues, resulting in reduced CP4 EPSPS protein expression in pollen (Table V-5). Specifically-timed glyphosate applications will produce a non-viable pollen phenotype through male tissue-specific glyphosate sensitivity in MON 87429 maize. Glyphosate is a systemic herbicide that is readily translocated via the phloem in plants (Devine et al., 1993). Once glyphosate is in the phloem, it moves to areas of high meristematic activity, following a typical source to sink distribution (Devine et al., 1993). Pollen development in a maize plant takes approximately four weeks to complete (Ma et al., 2008). Early tassel development begins at approximately V9 growth stage (Ritchie et al., 1997), therefore glyphosate applications made at around this time will allow maximum translocation of glyphosate to the male reproductive tissues, and selectively cause cell death only in those cells that are not tolerant to glyphosate (e.g., pollen cells).

The first-generation RHS product, MON 87427 maize, utilizes a specific promoter and intron combination (*e35S-hsp70*) to drive CP4 EPSPS expression in vegetative and female reproductive tissues. This specific promoter and intron combination in MON 87427 results in limited or no production of CP4 EPSPS protein in key male reproductive tissues; resulting in the non-viable pollen phenotype following late applications of glyphosate (USDA-APHIS Petition #10-281-01p p. 26). While the RHS trait in MON 87429 maize results in the same tissue-specific CP4 EPSPS expression seen in MON 87427, the mechanism by which this is achieved is enhanced in MON 87429.

Expression of CP4 EPSPS in MON 87429 plants is driven by the *CaMV 35S* promoter, a known constitutive promoter (Holtorf et al., 1995; Terada and Shimamoto, 1990). In maize and other monocot plants, *CaMV 35S* has been shown to drive weak gene expression in pollen tissue (Hamilton et al., 1992; Heck et al., 2005). Additionally, MON 87429 maize utilizes an endogenous maize regulatory element to target CP4 EPSPS mRNA for degradation specifically in tassel tissues, resulting in reduced CP4 EPSPS protein expression in pollen.

RNA interference (RNAi) is a natural process in eukaryotic organisms for the regulation of endogenous gene expression (Fire et al., 1998; Jones-Rhoades et al., 2006). Both microRNAs (miRNAs) and small interfering RNAs (siRNAs) can trigger the RNAi pathway (Carthew and Sontheimer, 2009). Endogenous maize male tissue specific small interfering RNAs (mts-siRNA) described in Yang et al. (2018), are expected to be involved in regulation of endogenous gene expression in male tissue such as the tassel. MON 87429 takes advantage of endogenous mts-siRNAs to degrade the CP4 EPSPS mRNA in male tissue. MON 87429 CP4 EPSPS mRNA contains a 201 bp siRNA Target Sequence in the 3' UTR (Figure IV-10) which is recognized by the endogenous mts-siRNAs resulting in degradation of the CP4 EPSPS mRNA, and reduced expression of CP4 EPSPS protein in male tissue.

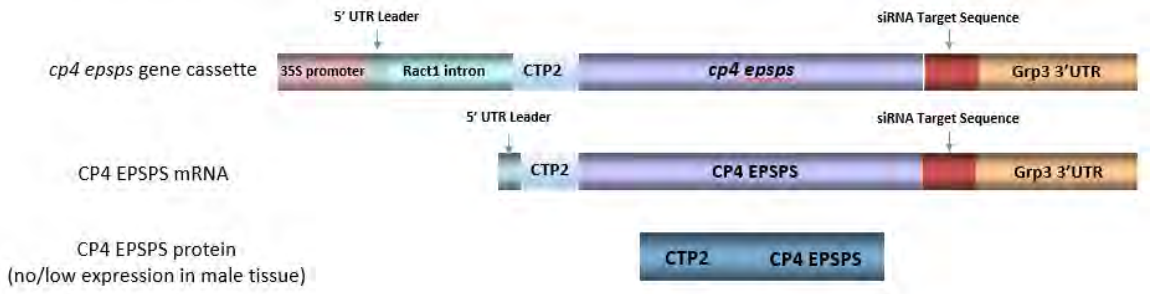


Figure IV-10. Schematic Diagram of *cp4 epsps* Gene Cassette, CP4 EPSPS mRNA and CP4 EPSPS Protein

The identification, cloning and testing of the 201 bp siRNA Target Sequence in MON 87429 is described in detail in Yang et al. (2018). The process is summarized as follows: 1) Identification of male tissue specific small interfering RNAs (mts-siRNA) primarily expressed in tassel, 2) Identification of Target Sequences corresponding to these endogenous mts-siRNAs present in maize, 3) Identification and selection of the target gene, EU974548, based on the 3' UTR region of this gene containing clustered, overlapping alignments of multiple siRNA Target Sequences, 4) Confirmation that the endogenous target, EU974548, is conserved across a wide range of maize germplasms, 5) Selection of the 201 bp siRNA Target Sequence, derived from EU974548, based on the intended non-viable pollen phenotype.

The model representing the mechanism of action by which mts-siRNAs trigger degradation of the CP4 EPSPS mRNA in MON 87429 tassel tissue is summarized below and shown in Figure IV-11. The first five steps represent the endogenous mts-siRNA pathway in conventional maize, based on current knowledge of siRNA driven RNAi systems (Gorski et al., 2017):

- Step 1: mts-siRNA precursor(s) RNA is produced from endogenous mts-siRNA gene.
- Step 2: The mts-siRNA precursor RNA is cleaved by the Dicer (DCL4) complex to produce mts-siRNAs.
- Step 3: Independently, endogenous target genes are transcribed that contain siRNA Target Sequences (shown as red line in the endogenous target gene double helix).
- Step 4: The resulting mts-siRNA molecules, from step 2, are then incorporated into multiprotein RNA-induced silencing complexes (RISC), which facilitate siRNA Target Sequence recognition and mRNA cleavage which leads to specific suppression of the target mRNA.
- Step 5: It is expected that endogenous mRNAs containing siRNA Target Sequences are cleaved by the RISC complex and then subjected to further degradation by 3'-5' and 5'-3' exoribonucleases due to the lack of a 3' poly adenine tail or a 5' cap structure at the cleavage site, inhibiting mRNA translation into protein.

The following three steps occur only in MON 87429 male tassel tissue as MON 87429 uses the endogenous siRNA machinery to specifically target CP4 EPSPS mRNA for degradation:

- Step 6: Upon CP4 EPSPS mRNA transcription, endogenous mts-siRNAs trigger cleavage of CP4 EPSPS mRNA via recognition of the 201bp siRNA Target Sequence in the 3' UTR of the CP4 EPSPS mRNA.
- Step 7: The CP4 EPSPS mRNA cleavage products are then subjected to degradation by 3'-5' and 5'-3' exoribonucleases due to the lack of a 3' poly adenine tail or a 5' cap structure at the cleavage site.
- Step 8: As a result of above processes, CP4 EPSPS mRNA is reduced, resulting in reduction in translation. Thus, little to no CP4 EPSPS protein is expressed in male tissue resulting in glyphosate sensitivity.

The same CP4 EPSPS mRNA is expressed throughout MON 87429 maize tissue (e.g., root, leaf, ear and tassel), however due to the male tissue specific expression of the mts-siRNAs, the CP4 EPSPS mRNA is only targeted for degradation in tassel tissue resulting in little to no expression of CP4 EPSPS protein in pollen (Table V-5).

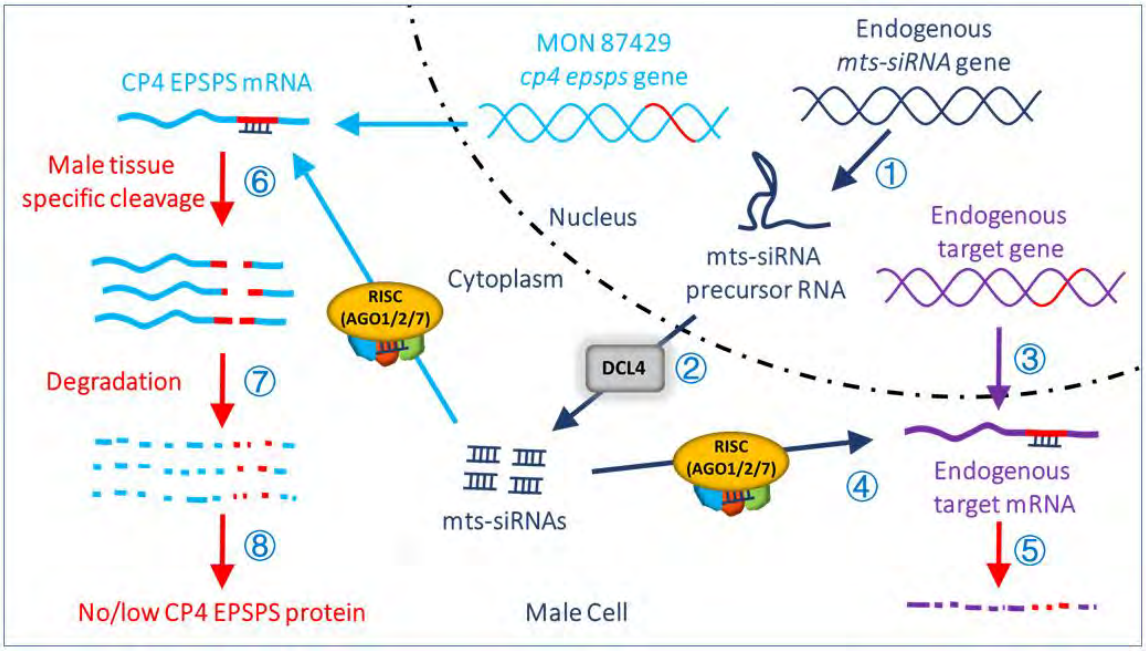


Figure IV-11. MON 87429 CP4 EPSPS siRNA Target Sequence Molecular Mechanism of Action

IV.H. Absence of Unintended Effects due to the MON 87429 RHS Mechanism of Action

IV.H.1. Lack of Secondary siRNA from CP4 EPSPS mRNA Suppression

In plants, primary mRNA degradation products, produced via siRNA mediated degradation, may be used as an RNA template for the generation of secondary siRNAs. These secondary siRNAs could initiate suppression of genes other than the intended target of the primary siRNA. Additionally, it has been reported that secondary siRNAs can move short distances (cell-to-cell) or long distances (between organs) from the site of origin and potentially silence genes with complementary sequence (Borges and Martienssen, 2015) resulting in the suppression of mRNAs other than the intended primary target. To determine whether secondary siRNAs are derived from the CP4 EPSPS transcript in MON 87429 plants, low molecular weight RNA Northern blotting was conducted (Figure IV-12). The upper panel shows high quality and equal loading of total RNA samples from various MON 87429 and conventional wildtype (WT) tissues. The RNA molecules were then transferred onto a membrane and hybridized with three probes that cover the entire CP4 EPSPS transcript except for the siRNA Target Sequence. The siRNA Target Sequence was intentionally excluded from the probes to avoid cross hybridization with the endogenous mts-siRNAs (Appendix C).

An *in vitro* synthesized 25-nt RNA oligo with a sequence identical to a portion of the CP4 EPSPS mRNA was detected as a positive control (Figure IV-12, middle left), indicating that the probes worked, and hybridization and visualization were successful. No hybridizing small RNAs were observed in any samples tested (including leaves, roots, tassels at different developmental stages, and ears) for either MON 87429 or the WT plants (Figure IV-12, middle), demonstrating that no secondary siRNAs were generated from the CP4 EPSPS transcripts in plants containing MON 87429. After stripping off the CP4 EPSPS probes, the blot was reprobed with a DNA oligo complementary to Zm-miRNA159, which is expressed in all maize tissues (Zhang et al., 2009) and used as a positive control here. Zm-miRNA159 was detected in all samples (Figure IV-12, bottom), indicating separation and electroblotting of small RNAs were successful (Yang et al., 2018).

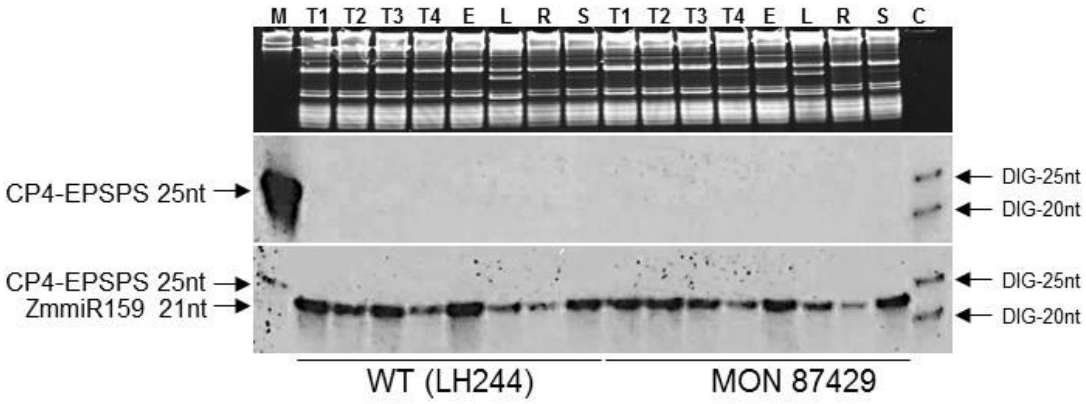


Figure IV-12. Absence of CP4 EPSPS Derived Secondary siRNAs.

Total RNA from various tissues of WT (LH244 conventional control; left side of blot) or MON 87429 maize (right side of blot), respectively, was used for Northern blotting. The upper panel shows equal loading and quality of the RNA samples by sharp, distinct RNA bands. M: a mix of the 1kb RNA ladder (1 µg) and a synthetic 25-nt CP4 EPSPS RNA oligo (1 ng) as a positive control; T1: V6 tassel; T2: V8 tassel; T3: V10 tassel; T4: V12 tassel; E: VT ear; L: V6 leaf; R: VE root; S: VE shoot; C: synthetic DIG-labeled 20- and 25-nt RNAs as small size markers. The middle panel is the low molecular weight RNA blot that was hybridized with three DIG-labeled probes covering the whole CP4 EPSPS transcript except the 201-bp target region. The lower panel is the same small RNA blot that was reprobed for miR159 after stripping off the CP4 EPSPS probes to demonstrate the quality of small RNA in the samples.

IV.H.1.1. Expression of Endogenous Genes with Sequence Homology to the siRNA Target Sequence

Bioinformatic analysis identified seven putative endogenous genes in the maize genome that contain sequences with at least 90% identity with the siRNA Target Sequence present in the CP4 EPSPS mRNA 3' UTR. All these putative genes are located in one region on chromosome 6. This is consistent with reports demonstrating that the maize genome is clustered with 60-80% highly repetitive sequences that are often non-functional pseudogenes and long terminal repeat (LTR) retrotransposons (Meyers et al., 2001; SanMiguel et al., 1996; Whitelaw et al., 2003). These putative genes share amino acid sequence homology with putative membrane proteins resembling serine incorporators that facilitate the synthesis of two serine-derived lipids, phosphatidylserine and sphingolipids (Yang et al., 2018). To confirm that the inclusion of the 201 bp siRNA Target Sequence in the *cp4 epsps* expression cassette did not impact the regulation of these seven putative endogenous genes, the expression levels of the putative gene transcripts in tassels were assessed.

Two quantitative reverse transcription PCR assays were developed: a TaqMan assay designed for detecting transcripts from two of the seven putative genes and a SYBR Green assay designed for six of the seven putative genes in the gene family (expression

of one putative gene can be detected by both assays) (Appendix C). Due to low transcript abundance and high sequence similarities between these seven putative genes, assays targeted to the individual transcripts could not be developed. Overall, these putative genes were expressed at very low levels in both transgenic MON 87429 and conventional wildtype (WT) control tassels. Furthermore, the expression levels of the putative endogenous genes in MON 87429 plants were comparable to those in WT control plants, and there were no significant differences in transcript expression between MON 87429 and WT control plants (Figure IV-13). Four additional representative conventional maize lines (Lines 1-4) were included to assess the natural variability in expression of these putative genes across conventional maize lines. The natural variability in expression of these putative genes proved to be greater than the difference between MON 87429 and WT control plants. These data demonstrate there is no impact to the regulation of the seven putative genes that share sequence homology with the siRNA Target Sequence in MON 87429. Furthermore, there were no observed off-type phenotypes in MON 87429 maize plants (e.g. flowering, pollen characteristics or grain yield) that would be indicative of adverse unintended effects (Section VII).

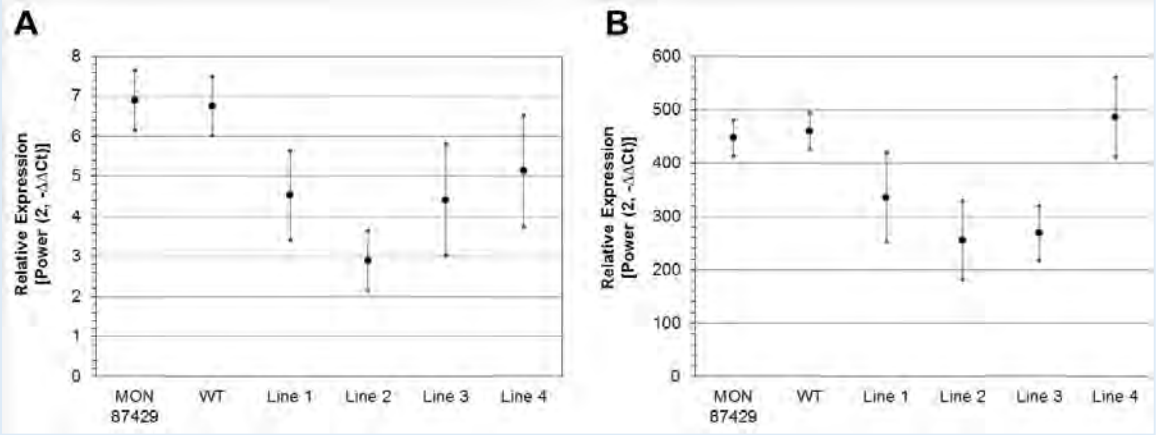


Figure IV-13. Expression of Endogenous Genes with Sequence Homology to the siRNA Target Sequence in Tassels

(A) Expression levels of two endogenous genes as determined by a TaqMan assay. (B) Expression levels of six endogenous genes as determined by a SYBR Green assay. Plants containing MON 87429 (test) and WT (LH244 conventional control) and additional four conventional maize lines (reference) were grown in a randomized complete block design with 100 replications for the test and control materials, and 25 replications for each reference material in a greenhouse. Each replication included three technical replicates (subsamples) per material. Real-time PCR was conducted in triplicate for each subsample. The statistical analysis using a linear mixed model for a randomized complete block design reveals there are no significant differences between test and WT control at the 5% level. Large dots: mean relative expression level; small dots: upper or lower 95% confidence interval, respectively.

IV.I. Characterization of the Genetic Modification Summary and Conclusion

As described above, characterization of the genetic modification in MON 87429 was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterization demonstrate that MON 87429 contains a single copy of the intended T-DNA containing the *pat, dmo, ft_t,* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions are based on the following:

- Molecular characterization of MON 87429 by Next Generation Sequencing (NGS) demonstrated that MON 87429 contains a single intended DNA insert. These whole-genome analyses provided a comprehensive assessment of MON 87429 to determine the presence and identity of sequences derived from PV-ZMHT519224 and demonstrated that MON 87429 contains a single T-DNA insert with no detectable plasmid backbone sequences.

- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) performed on MON 87429 was used to determine the complete sequence of the single DNA insert from PV-ZMHT519224, the adjacent flanking genomic DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the sequence and organization of the DNA is identical to the corresponding region in the PV-ZMHT519224 T-DNA. Furthermore, the genomic organization at the insertion site in MON 87429 was assessed by comparing the sequences flanking the T-DNA insert in MON 87429 to the sequence of the insertion site in conventional maize. This analysis determined that 54 bases were deleted upon T-DNA integration. There also was a 29 base insertion in the MON 87429 5′ flanking sequence and a 31 base insertion in the MON 87429 3′ flanking sequence.

- Generational stability analysis by Next Generation Sequencing (NGS) demonstrated that the single PV-ZMHT519224 T-DNA insert in MON 87429 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 87429.

- Segregation analysis corroborates the insert stability demonstrated by NGS and independently establishes the nature of the T-DNA as a single chromosomal locus that shows an expected pattern of inheritance.

- MON 87429 maize utilizes an endogenous maize regulatory element to target CP4 EPSPS mRNA for degradation in tassel tissues, resulting in reduced CP4 EPSPS protein expression in pollen with no impact on endogenous plant gene expression.

Taken together, the characterization of the genetic modification in MON 87429 demonstrates that a single copy of the intended T-DNA was stably integrated at a single

locus of the maize genome and that no plasmid backbone sequences are present in MON 87429.

In addition, MON 87429 maize utilizes an endogenous maize regulatory element (an siRNA Target Sequence) to target CP4 EPSPS mRNA for degradation in tassel tissues. A focused study on the MON 87429 siRNA Target Sequence demonstrated the absence of unintended effects on endogenous gene regulation.

V. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE DMO, PAT, CP4 EPSPS AND FT_T PROTEINS PRODUCED IN MON 87429

Characterization of the introduced protein(s) in a biotechnology-derived crop is important to establishing food, feed, and environmental safety. As described in Section IV, MON 87429 contains *dmo, pat, cp4 epsps* and *ft_t* expression cassettes that, when transcribed and translated, result in the expression of the DMO, PAT, CP4 EPSPS and FT_T proteins, respectively. Based on the previous characterizations and safety assessments, as well as the history of safe use for the DMO, PAT and CP4 EPSPS proteins, information pertaining to the characterization, equivalence, allergenicity and toxicity of these proteins will not be discussed in detail in this petition. Summaries of safety assessments for DMO, PAT, and CP4 EPSPS proteins that have been previously evaluated by the USDA-APHIS are found in Sections V.A., V.B. and V.C. The characterization and safety assessment of the FT_T protein is found in section V.D. These assessments support a conclusion that the DMO, PAT, CP4 EPSPS, and FT_T proteins produced in MON 87429 are safe for human and animal consumption and do not impact the plant pest risk of MON 87429 maize.

V.A. DMO Protein Safety Assessment Summary

MON 87429 maize contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein. As a mono-oxygenase protein, the DMO protein is part of a the larger oxygenase family of enzymes that incorporate one or two oxygen atoms into substrates and are widely distributed in many universal metabolic pathways (Harayama et al., 1992). In MON 87429 maize, the DMO protein enzymatically catalyzes the demethylation of the broadleaf herbicide dicamba to the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) and formaldehyde, thus conferring dicamba resistance (Chakraborty et al., 2005). Expression of the DMO protein in MON 87429 is targeted to the chloroplast by a chloroplast transit peptide (CTP), which facilitates its co-localization with the endogenous reductase and ferredoxin enzymes required to supply electrons for the DMO demethylation reaction (Behrens et al., 2007). The DCSA product of the reaction catalyzed by the DMO protein is a known metabolite of dicamba in conventional cotton, soybean, soil, and livestock, and its safety has been evaluated by the FAO-WHO and EPA (FAO-WHO, 2011a; U.S. EPA, 2009). The other reaction product, formaldehyde, is found naturally in many plants and edible fungi at levels up to several hundred ppm (Adrian-Romero et al., 1999; Tashkov, 1996).

In the construction of the plasmid vector used in the development of MON 87429, PV-ZMHT519224, the *Albino and pale green 6* chloroplast transit peptide (CTP) coding sequence from *Arabidopsis thaliana* (*APG6*, Table IV-1) was joined to the *dmo* coding sequence, resulting in the production of a precursor protein consisting of the DMO protein and an N-terminal 68 amino acid chloroplast transit peptide APG6, which is used to target the precursor protein to the chloroplast (Herrmann, 1995; Klee et al., 1987). Typically, transit peptides are precisely removed from the precursor protein following delivery to the targeted plastid (della-Cioppa et al., 1986) resulting in the full length protein. However, there are examples in the literature of alternatively processed forms of

a protein targeted to a plant's chloroplast (Behrens et al., 2007; Clark and Lamppa, 1992). Data from N-terminal sequencing analysis of the MON 87429-produced DMO indicate that processing of the DMO precursor protein expressed in MON 87429 produced two isoforms of the mature MON 87429 DMO protein that differ by a single, additional amino acid at the N-terminus that is derived from the CTP. The single amino difference between the two isoforms of the DMO protein expressed in MON 87429 (designated DMO+1 and DMO+0) results in the presence of two polypeptide chains of 341 and 340 amino acids, respectively, with an apparent molecular weight ~38 kDa, as the two forms are indistinguishable by Coomassie stain and western blot analysis of SDS-PAGE gels. Thus, MON 87429 DMO protein will be used to refer to both forms of the protein collectively in this petition, except where stated otherwise.

The MON 87429 DMO protein shares a high level of sequence identity with DMO proteins previously assessed and present in biotechnology-derived crops that were deregulated by USDA-APHIS (MON 87708 soybean, USDA-APHIS Petition #10-188-01p, MON 88701 cotton, USDA-APHIS Petition #12-185-01p and MON 87419 maize, USDA-APHIS Petition #15-113-01p). Additionally, these biotechnology-derived crops expressing DMO proteins completed consultation with U.S. FDA (MON 87708 soybean, BNF 000125, MON 88701 cotton, BNF 000135 and MON 87419 maize, BNF 000135), where it was demonstrated that food and feed derived from these events are not materially different than the respective conventional crops. The minor amino acid substitutions between the wild-type DMO protein from the DI-6 strain of *S. maltophilia*, the MON 87429 DMO protein and the DMO proteins expressed in these other biotechnology-derived crops are localized to the N-terminus of the proteins and at position 112 of the amino acid sequences (Herman et al., 2005) (Figure V-1). Additionally, based upon the crystal structure of the wild-type DMO proteins, these amino acid substitutions are structurally distant from the active site and are not expected to impact catalytic site coordination, functional activity, immunoreactivity or specificity (D'Ordine et al., 2009; Dumitru et al., 2009; Wang et al., 2016). Thus, prior safety assessments of the DMO protein expressed in other biotechnology-derived crops are directly applicable to the DMO protein expressed in MON 87429.

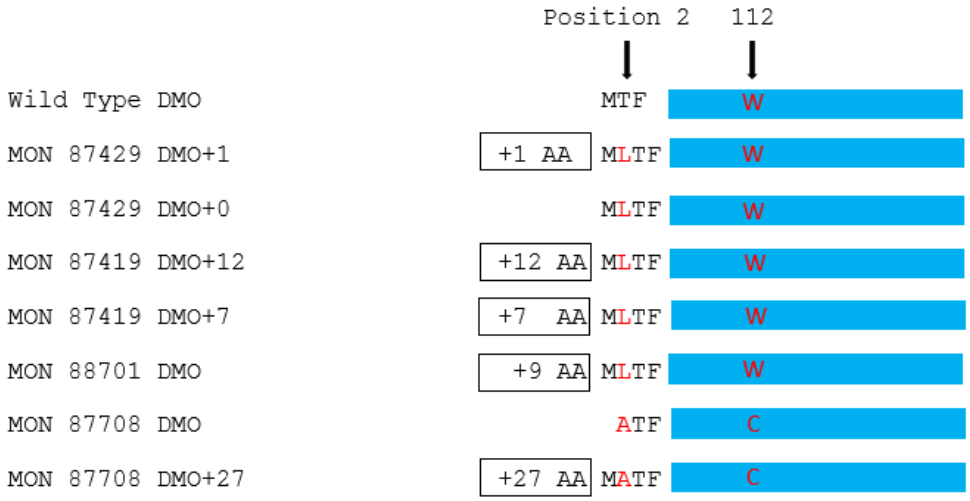


Figure V-1. Forms of DMO Protein and Their Relation to the Wild-Type DMO Protein

The diagram represents the various DMO forms discussed in this section. Position refers to amino acid residues as wild-type DMO and the N-terminal boxed region indicates residues from CTPs. The blue regions indicate regions of 100% amino acid identity. The wild-type DMO form isolated from *S. maltophilia* was the first form sequenced (Herman et al., 2005). The MON 87429 DMO proteins are identical to wild-type DMO, except for the insertion of a leucine at position 2 and an addition of 1 amino acid encoded by the *AGP6* gene at the N-terminus for MON 87429 DMO+1. The MON 87419 DMO proteins are identical to wild-type DMO, except for the insertion of a leucine at position 2 and the amino acids derived from the C-terminal transit peptides at the N-terminus, which are 12 and 7 amino acids encoded by the *CTP4* gene at the N-terminus for MON 87419 DMO+12 and MON 87419 DMO+7, respectively. The MON 88701 DMO protein is identical to wild-type DMO, except for an insertion of a leucine at position 2, and an addition of 9 amino acids encoded by the *CTP2* gene at the N-terminus. The MON 87708 DMO proteins are identical to wild-type DMO, except for the insertion of an alanine at position 2, a single amino acid change at position 112 (tryptophan to cysteine) and an additional 27 amino acids encoded by the *RbcS* gene at the N-terminus for MON 87708 DMO+27. The MON 87708 DMO (fully processed) protein additionally lacks a lead methionine residue.

The DMO protein is specific for the oxidative demethylation of dicamba, forming DCSA. Dicamba interacts with amino acids in the catalytic site of DMO through both the carboxylate moiety and the chlorine atoms of dicamba, which are primarily involved in orienting the substrate in the catalytic site. These chlorine atoms are required for catalysis (D'Ordine et al., 2009; Dumitru et al., 2009). Given the limited existence of chlorinated compounds with structures similar to dicamba in plants and other eukaryotes (Wishart, 2010; Wishart et al., 2009), it is unlikely that MON 87429 DMO will catalyze the conversion of endogenous compounds. In order to confirm the specificity of the MON 87429 DMO protein for dicamba, and to demonstrate that the minor differences in amino acid sequences present in the MON 87429 DMO protein relative to the DMO proteins expressed in previous biotechnology-derived crops protein do not impact the activity or selectivity for dicamba herbicide as compared to potential endogenous substrates, the potential for MON 87429 DMO to catabolize dicamba and *o*-anisic acid was evaluated using the same qualitative assay used to evaluate the selectivity of

MON 87708 DMO (USDA-APHIS Petition #10-188-01p p. 76). Although *o*-anisic acid is not known to be present in corn, this substance was chosen for this confirmatory experiment since, among the five substrates used in the original study, which included ferulic acid, *o*-anisic acid, sinapic acid, syringic acid, and vanillic acid, *o*-anisic acid is the substrate that is most structurally similar to dicamba. The results from this assessment were similar to the previously reported results for MON 87708 DMO in that the DCSA product was observed using dicamba as the substrate whereas no demethylated products were observed with *o*-anisic acid as the substrate (Appendix E), confirming that the MON 87429 DMO did not catabolize *o*-anisic acid. Thus, MON 87429 DMO is active and has a high specificity for dicamba as a substrate.

The data and information summarized in this section confirm that the molecular mechanism of the MON 87429 DMO protein that confers dicamba tolerance is well understood, that the MON 87429 DMO protein is structurally and functionally homologous to the DMO proteins present in biotechnology-derived crops that have been deregulated by USDA-APHIS and demonstrated to not be materially different than the respective conventional crops in consultation with the US FDA, and that the MON 87429 DMO protein is specific for dicamba. Thus, prior environmental and safety assessments for the DMO protein demonstrating the lack of impact on plant pest potential, the lack of homology to known protein toxins or allergens, digestibility in *in vitro* digestion assays and lack of acute oral toxicity are directly applicable to the MON 87429 DMO protein and are not detailed further herein.

V.B. PAT Protein Safety Assessment Summary

MON 87429 maize contains an acetyltransferase gene from *Streptomyces viridochromogenes* that expresses phosphinothricin N-acetyltransferase (PAT) protein. The molecular mechanism of the PAT protein, which acetylates glufosinate in the presence of acetyl CoA to form N-acetyl glufosinate, is well understood (Thompson et al., 1987). Glufosinate is a racemic mixture of the D- and L-forms of the amino acid phosphinothricin. The herbicidal activity of glufosinate results from the binding of L-phosphinothricin to glutamine synthetase (OECD, 1999; 2002a). Expression of the PAT protein in MON 87429 maize results in the ability to covert L-phosphinothricin to the non-herbicidal N-acetyl glufosinate, thus conferring glufosinate resistance to the crop.

Phosphinothricin N-acetyltransferase (PAT) proteins have been isolated from two separate species of *Streptomyces*, *S. hygroscopicus* (Thompson et al., 1987) and *S. viridochromogenes* (Wohlleben et al., 1988). The PAT protein isolated from *S. hygroscopicus* is encoded by the *bar* gene, and the PAT protein isolated from *S. viridochromogenes* is encoded by the *pat* gene. These PAT proteins are made up of 183 amino acids with 85% identity to each other at the amino acid level (Wohlleben et al., 1988). Based on previous studies (Wehrmann et al., 1996) that have extensively characterized PAT proteins produced from *bar* and *pat* genes, OECD recognizes both proteins to be equivalent with regard to function and safety (OECD, 1999). Expression of the *pat* gene in MON 87429 results in a single polypeptide of 182 amino acids with an apparent molecular weight of ~25 kDa. Data from N-terminal sequencing analysis of the MON 87429-produced PAT protein indicate that it is identical to the wild type PAT

protein encoded by *S. viridochromogenes* and to the PAT proteins produced in several commercially available glufosinate tolerant crops, including events T25, TC1507, A2704-12, A5547-127 and DAS-59122-7 (USDA-APHIS Petitions #94-357-01p, #00-136-01p, #96-068-01p, #98-014-1p, #03-353-01p respectively) (Hérouet et al., 2005; ILSI-CERA, 2011), except for the first methionine that is removed due to co-translational processing in MON 87429. N-terminal methionine cleavage is common and naturally occurs in the vast majority of proteins (Meinnel and Giglione, 2008).

Numerous glufosinate-tolerant crops including maize, canola, soybean, sugar beet, rice and cotton have been deregulated by USDA-APHIS (USDA-APHIS Petitions #94-357-01p, #00-136-01p, #03-353-01p and #15-113-01p maize; #98-278-01p and 01-206-01p canola, #98-014-01p, #12-215-01p, #11-234-01p and #09-349-01p soybean; #97-336-01p sugar beet, #98-329-01p rice; and #12-185-01p, #13-262-01p and #08-340-01p cotton) and completed consultations with US FDA (U.S. FDA, 1995b; a; 1996; 1997; 1998a; b; 1999; 2002), where it was demonstrated that food and feed derived from these crops are not materially different than the respective conventional crops. The safety of PAT proteins has been confirmed following extensive reviews by regulatory agencies in at least 15 different countries for more than 30 biotechnology-derived events in several different crop species (e.g., maize, soybean, cotton, canola and sugar beet). Additionally, the EPA has issued a tolerance exemption for PAT protein (U.S. EPA, 1997). Prior safety assessments of the PAT proteins expressed in these other biotechnology-derived crops are directly applicable to the MON 87429 PAT protein because the amino acid sequence of the MON 87429 PAT protein is identical to the PAT proteins in these biotechnology-derived crops that are derived from the *pat* gene and because the PAT proteins produced from the *bar* and *pat* genes are equivalent in terms of function and safety. The PAT proteins have a robust history of safe consumption and safe use in agriculture that is supported by the lack of any documented reports of adverse human or animal affects since the introduction of biotechnology-derived crops expressing PAT proteins in 1995 (Duke, 2005).

The PAT protein expressed in MON 87429 is highly specific for glufosinate. Enzyme assays have demonstrated that the PAT protein is unable to acetylate other common L-amino acids that are structurally similar to L-phosphinothricin, and substrate competition assays showed no inhibition of glufosinate acetylation in the presence of high concentrations of L-amino acids that are structurally similar to L-phosphinothricin (including the glufosinate analog L-glutamate) (Wehrmann et al., 1996). Recent metabolic profiling reported non-specific PAT (*bar*) mediated acetylation of two amino acids (aminoadipate and tryptophan) in senescent leaf extracts from *A. thaliana*, however this observation has not been reported in maize (Christ et al., 2017). Thus, the PAT protein has high substrate specificity for L-phosphinothricin, the herbicidal component of glufosinate, and it has been shown in other PAT-expressing maize products (e.g., T25, TC1507, MON 87419 and DAS-59122-7) that PAT does not affect maize metabolism.

The data and information summarized in this section confirm that the molecular mechanism of the MON 87429 PAT protein that confers glufosinate tolerance is well understood, that the MON 87429 PAT protein is identical to the PAT proteins present in biotechnology-derived crops that have been deregulated by USDA-APHIS and

demonstrated to not be materially different than the respective conventional crops in consultation with the US FDA, that the MON 87429 PAT protein is highly specific for glufosinate and that the PAT protein does not affect maize metabolism. Thus, prior environmental and safety assessments for the PAT protein demonstrating lack of impact on plant pest potential, lack of homology to known protein toxins or allergens, digestibility in *in vitro* digestion assays and lack of acute oral toxicity are directly applicable to the MON 87429 PAT protein and are not detailed further herein.

V.C. CP4 EPSPS Protein Safety Assessment Summary

MON 87429 contains a 5-enolpyruvulshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium* sp. strain CP4 (*cp4 epsps*) that expresses a EPSPS protein. EPSPS is a key enzyme involved in aromatic amino acid biosynthesis and catalyzes a reaction where the enolpyruvyl group from phosphoenol pyruvate (PEP) is transferred to the 5-hydroxyl of shikimate-3-phosphate (S3P) to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Alibhai and Stallings, 2001). Shikimic acid is a substrate for the biosynthesis of aromatic amino acids (phenylalanine, tryptophan and tyrosine) and other aromatic molecules that are necessary for plant growth. The shikimic acid pathway and EPSPS enzymes are ubiquitous to plants and microorganisms, but are absent in mammals, fish, birds, reptiles, and insects (Alibhai and Stallings, 2001). The CP4 EPSPS protein expressed in MON 87429 is structurally similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Sikorski and Gruys, 1997). In conventional plants, glyphosate blocks the biosynthesis of EPSP, thereby depriving plants of essential amino acids (Haslam, 1993; Steinrücken and Amrhein, 1980). In Roundup Ready® plants, which are tolerant to Roundup® agricultural herbicides, requirements for aromatic amino acids and other metabolites are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette et al., 1996).

The *cp4 epsps* expression cassette in MON 87429 encodes a precursor protein of 531 amino acids (455 amino acids encoded by the *cp4 epsps* gene and 76 amino acids encoded by the *CTP2* gene for targeting the CP4 EPSPS protein into chloroplasts). Expression of the *cp4 epsps* gene in MON 87429 results in a single polypeptide chain of 455 amino acids with an apparent molecular weight of ~44 kDa. Data from N-terminal sequencing analysis of the MON 87429-produced CP4 EPSPS protein indicate that the polypeptide chain starts at methionine position 77 after complete cleavage of the chloroplast transit peptide (CTP2) (Padgette et al., 1996). The CP4 EPSPS protein expressed in MON 87429 is identical in structure and function to the CP4 EPSPS protein expressed in Roundup Ready® products across several crops, including soybeans, maize, canola, cotton, sugar beet, and alfalfa.

The safety and mode-of-action of CP4 EPSPS protein is well documented and is the subject of many publications (Harrison et al., 1996; Hoff et al., 2007; ILSI-CERA, 2010; U.S. EPA, 1996a). Numerous glyphosate-tolerant, commercially available CP4 EPSPS containing crops have been deregulated by USDA-APHIS (USDA-APHIS Petitions #10-281-01p and 04-125-01p maize; 11-188-01p and 98-216-01p canola; 04-110-01p

alfalfa; 06-178-01p and 93-258-01p soybean) and have completed consultations with the FDA (e.g., MON 87427 BNF 000126 in 2012 and MON 88017 BNF 000097 in 2005), where it was demonstrated that food and feed derived from these crops are not materially different than the respective conventional crops. The safety of the CP4 EPSPS protein has been reviewed by regulatory agencies around the world (ILSI-CERA, 2011; OECD, 1999; 2002a). Additionally, in 1996 the U.S. EPA established an exemption from the requirement of a tolerance for residues of the plant pesticide inert ingredient CP4 EPSPS and the genetic material necessary for its production in all plants (40 CFR § 174.523, redesignated from § 180.1174, effective April 25, 2007). Prior safety assessments of the CP4 EPSPS protein expressed in these other biotechnology-derived crops are directly applicable to the MON 87429 CP4 EPSPS protein because the amino acid sequence and function of the MON 87429 CP4 EPSPS protein is identical to the CP4 EPSPS proteins in these biotechnology-derived crops. The CP4 EPSPS protein has a robust history of safe consumption and safe use in agriculture that is supported by the lack of any documented reports of adverse human or animal effects since the introduction of biotechnology-derived crops expressing CP4 EPSPS protein.

EPSPS enzymes, including the MON 87429 CP4 EPSPS protein, are highly specific for their substrates. The only known substrates of any biological significance for EPSPS enzymes are S3P and PEP. Glyphosate is not enzymatically modified by EPSPS. Shikimic acid was shown to be a very poor substrate for EPSPS enzyme, requiring much higher concentrations to observe turnover by the enzyme than for S3P (Gruys et al., 1992). Methyl shikimate, quinic acid, and dihydroshikimic acid do not serve as substrates for the EPSPS enzyme (Franz et al., 1997). As with most physiological pathways, there is tight regulation of metabolic flux through the shikimic acid pathway. Pathway flux is regulated both transcriptionally and post-transcriptionally (Maeda and Dudareva, 2012; Tzin et al., 2012). The first enzyme in the pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), has been identified as the key regulatory checkpoint for the flux through the pathway, with possible secondary checkpoints at shikimate kinase and chorismate synthase (Maeda and Dudareva, 2012; Tzin et al., 2012). Thus, plants, have mechanisms to regulate flux through the shikimate pathway irrespective of EPSPS synthase activity levels. Due to both the high substrate specificity of EPSPS enzymes and lack of a role as a regulatory enzyme in the shikimic acid pathway, there is no plausible mechanism for the modification of endogenous plant constituents due to the expression of CP4 EPSPS.

The data and information summarized in this section confirm that the molecular mechanism of the MON 87429 CP4 EPSPS protein that confers glyphosate tolerance is well understood, that the MON 87429 CP4 EPSPS protein is identical to the CP4 EPSPS proteins present in biotechnology-derived crops that have been deregulated by USDA-APHIS and demonstrated to not be materially different than the respective conventional crops in consultation with the US FDA, that the MON 87429 CP4 EPSPS protein is selective for glyphosate and that the CP4 EPSPS protein does not affect maize metabolism. Thus, prior environmental and safety assessments for the CP4 EPSPS protein demonstrating a lack of impact on plant pest potential, lack of homology to known protein toxins or allergens, digestibility in *in vitro* digestion assays and lack of

acute oral toxicity are directly applicable to the MON 87429 CP4 EPSPS protein and are not detailed further herein.

V.D. FT_T Protein Characterization and Safety Assessment

A multistep approach to the safety assessment of the MON 87429 FT_T proteins was conducted according to guidance established by the Codex Alimentarius Commission (Codex Alimentarius, 2009) and OECD, which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties. The assessment includes: 1) documenting the history of safe consumption of the expressed protein or its structural and functional homology to proteins that lack adverse effects on human or animal health; 2) characterization of the physicochemical and functional properties of each expressed protein; 3) quantification of each expressed proteins' expression in plant tissues; 4) examination of the similarity of each expressed protein to known allergens, toxins or other biologically active proteins known to have adverse effects on humans and animals; 5) evaluation of the susceptibility of each expressed protein to the digestive enzymes pepsin and pancreatin; 6) a confirmatory evaluation of potential animal toxicity through an animal assay. The safety assessment supports the conclusion that exposure to the FT_T protein derived from MON 87429 would not pose any meaningful risk to human or animal health or the environment.

V.D.1. Identity and Function of the FT_T protein from MON 87429

The FT_T protein produced in MON 87429 is encoded by the *ft_t* gene that provides tolerance to aryloxyalkanoate herbicides. Aryloxyalkanoate herbicides include the aryloxyphenoxypropionate acetyl coenzyme A carboxylase (ACCase) inhibitors (so called "FOPs" herbicides such as quizalofop) and some synthetic auxins, such as 2,4-D. The *ft_t* gene in MON 87429 is a modified version of the R-2,4-dichlorophenoxypropionate dioxygenase (*Rdp*a) gene from the soil bacteria *Sphingobium herbicidovorans*. The amino acid sequence of the FT_T protein shares ~ 89% sequence identity with wild type RdpA (Figure V-2). A total of 30 amino acid substitutions throughout the protein sequence resulted in FT_T displaying improved enzyme kinetics (increased V_{max}) and substrate affinity (reduced K_m) for 2,4-D, relative to RdpA (Appendix G, Table G-1). The amino acid modifications also resulted in FT_T protein displaying retained activity at temperatures experienced during the summer months in maize growing areas compared to RdpA protein (Appendix G, Table G-1).

```
1    AMHAALTPLT NKYRFIDVQP LTGVLGAEIT GVDLREPLDD STWNEILDAF
51   HTYQVIYFPG QAITNEQHIA FSRRFGPVDP VPILKSIEGY PEVQMIRREA
101  NESSRFIGDD WHTDSTFLDA PPAAVVMRAI EVPEYGGDTG FLSMYSAWET
151  LSPTMQATIE GLNVVHSATK VFGSLYQATN WRFSNTSVKV MDVDAGDRET
201  VHPLVVTHPV TGRRALYCNQ VYCQKIQGMT DAESKSLLQF LYEHATKFDF
251  TCRVRWKKDQ VLVWDNLCTM HRAVPDYAGK FRYLTRTTVA GDKPSR
```

Figure V-2 Deduced Amino Acid Sequence of the FT_T Protein

The amino acid sequence of the MON 87429 FT_T protein was deduced from the full-length coding nucleotide sequence present in PV-ZMHT519224 (See Table III-1 for more detail). The double underline shows the alanine amino acid from the CTP MDH that is the N-terminus of the mature FT_T protein. The single underline shows the 30 amino acid substitutions in FT_T. The number counts start from methionine corresponding to start codon. The substitutions are S6T, S9T, Q10N, R11K, F12Y, E13R, R14F, A16D, L82I, G103S, V105F, D130E, H134Y, T145S, R169K, Q178T, R180W, G209V, S210T, K213R, G214A, V217C, R224K, E226Q, P235S, R246K, G289A, V291D, R292K, and A294S, where the first letter denotes the original amino acids followed by the position and the new amino acid.

RdpA protein is an alpha-ketoglutarate-dependent non-heme iron dioxygenase (Müller et al., 2006), and given their structural similarity, the FT_T protein is also an alpha-ketoglutarate-dependent non-heme iron dioxygenase. Alpha-ketoglutarate-dependent non-heme iron dioxygenases belong to a diverse superfamily of Fe(II)/alpha-ketoglutarate dependent hydroxylases that catalyze a range of oxygenation reactions in synthesis and decomposition reactions that include hydroxylation reactions, desaturations, demethylations, ring expansions, ring formations and other oxidative reactions (Hausinger, 2004). This protein superfamily is broadly distributed across the plant, animal and bacterial kingdoms, therefore environmental exposure to Fe(II)/alpha-ketoglutarate dependent hydroxylases is ubiquitous. Members of this superfamily share a common double-stranded, beta-helix protein fold with three metal-binding ligands found in a His1-X-Asp/Glu-Xn-His2 motif (Hausinger, 2004). In oxygenation reactions, alpha-ketoglutarate (αKG) chelates Fe(II) using its C1 carboxylate and C2-ketone. Decarboxylation of αKG results in the formation of succinate and carbon dioxide, which leads to the generation of an Fe(IV)-oxo or other activated oxygen species that subsequently hydroxylate the primary substrate, e.g., quizalofop (Bugg, 2003; Carolis and Luca, 1994; Hausinger, 2004). Thus, the FT_T protein catalyzes a dioxygenase reaction in the presence of αKG and oxygen to metabolize quizalofop, a FOP herbicide, into the herbicidally-inactive quizalofop phenol and pyruvate (Figure V-3). The FT_T protein also catalyzes the dioxygenase reaction that degrades 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin herbicide, into herbicidally-inactive 2,4-dichlorophenol (2,4-DCP) and glyoxylic acid in the presence of alpha-ketoglutarate and oxygen. Succinate and carbon dioxide are released as products of this reaction (Figure V-3). The safety of 2,4-D, quizalofop, and their relevant metabolites

have been assessed by US EPA. US EPA concluded that there is a reasonable certainty that no harm will result to the general population, or to infants and children from aggregate exposure to 2,4-D (U.S. EPA, 2017a) or quizalofop (U.S. EPA, 2018) residues or their metabolites.

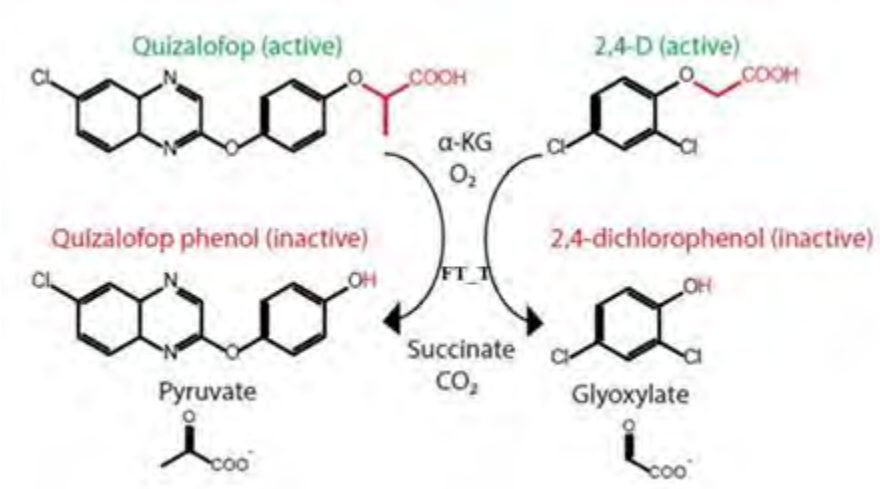


Figure V-3. Substrate and Metabolites of FT_T Protein Reaction with Quizalofop (left) and 2,4-D (right)

V.D.2. Characterization and Equivalence of FT_T protein from MON 87429

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of the introduced protein(s) produced from the inserted DNA and accompanied by various assessments to confirm the safety of the protein(s). For safety data generated using *E. coli*-produced protein(s) to be applied to plant-produced protein(s), the equivalence of the plant- and *E. coli*-produced proteins must be assessed.

The MON 87429-produced FT_T protein purified from grain of MON 87429 was characterized and the equivalence of the physicochemical and functional properties between the MON 87429-produced FT_T and *E. coli*-produced MON 87429 FT_T proteins was established using a panel of analytical tests: 1) N-terminal sequence analysis of MON 87429-produced FT_T determined the expected N-terminal sequence; 2) Nanoscale liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 87429-produced FT_T sequence; 3) western blot analysis with an antibody specific for FT_T protein demonstrated that the immunoreactive properties of the MON 87429-produced FT_T and *E. coli*-produced MON 87429 FT_T were equivalent; 4) SDS-PAGE analysis showed that the electrophoretic mobility and apparent molecular weight of the MON 87429-produced FT_T and *E. coli*-produced MON 87429 FT_T proteins were equivalent; 5) MON 87429-produced FT_T and *E. coli*-produced MON 87429 FT_T proteins were both determined to be non-glycosylated; and 6) functional activity analysis demonstrated that MON 87429-produced FT_T and

E. coli-produced MON 87429 FT_T proteins had equivalent enzymatic activity (See Appendix D).

Taken together, these data provide a detailed and thorough characterization of the MON 87429-produced FT_T protein and establish its equivalence to *E. coli*-produced MON 87429 FT_T protein. This equivalence justifies the use of the *E. coli*-produced MON 87429 FT_T protein in studies to assess the safety of the FT_T protein expressed in MON 87429.

V.D.3. FT_T Protein Donor Organism and History of Safe Use

MON 87429 contains the *ft_t* gene, a modified version of the *Rdpa* gene from *Sphingobium herbicidovorans*, that expresses the FT_T protein. FT_T is a modified version of the R-2,4-dichlorophenoxypropionate dioxygenase (RdpA) protein (Müller et al., 2006). *S. herbicidovorans* is a common gram-negative, rod-shaped, non-motile, non-spore-forming soil bacterium (Takeuchi et al., 2001; Zipper et al., 1996), which is strictly aerobic and chemo-organotrophic, and not known to be associated with human disease. The taxonomy of *S. herbicidovorans* is:

Kingdom: Bacteria
Phylum: Proteobacteria
Class: *Alphaproteobacteria*
Order: *Sphingomonadales*
Family: *Sphingomonadaceae*
Genus: *Sphingobium*

Members of the genus *Sphingobium* have been isolated from a wide variety of habitats including soil and freshwater (Chaudhary et al., 2017). *Sphingobium* species have also been isolated from foods such as corn (Rijavec et al., 2007), papaya (Thomas et al., 2007) and tomato (Enya et al., 2006). Thus, there is wide-spread human and animal exposure to the *Sphingobium* species in the environment without any known adverse safety or allergenicity reports.

As noted in Section V.D.1, the FT_T protein shares the common high order structure of alpha-ketoglutarate-dependent dioxygenases that contains a classical dioxygenase active pocket including an iron atom coordinated by two histidine residues and one aspartic acid or glutamate residue (Hausinger, 2004). These alpha-ketoglutarate-dependent dioxygenases have been identified in a broad range of organisms including bacteria, fungi, plants, and vertebrates, which have been extensively consumed by both humans and animals (Hausinger, 2004; Kundu, 2012) without any reports of adverse effects.

V.D.4. FT_T Protein Catalyzes a Specific Enzyme Reaction

Whereas the amino acid modifications described in Section V.D.1 present in the FT_T protein, relative to wild type RdpA from *S. herbicidovorans*, improved enzymatic activity, substrate affinity and retained activity at increased temperatures, they did not alter the specificity of the FT_T protein for its substrate. To confirm FT_T protein substrate specificity was not impacted by the optimization, an endogenous substrate

specificity assessment was conducted. Endogenous plant small molecules with similar structures to known FT_T substrates, which are compatible with the FT_T enzymatic active site, were investigated as potential substrates.

The screening approach, detailed in Appendix G, utilized a three-step process: Step 1, the NAPRALERT[8] database, which includes plant specific small molecule datasets collected from several crop plant species including corn (Bisson et al., 2016), was utilized to identify small molecules with structural similarity to dichlorprop, a synthetic auxin herbicide that has the most basic structure of the herbicidal aryloxyalkanoate compounds. In Step 2, selected compounds were then subjected to *in silico* protein-small molecule docking simulations using the structure of the coordinated FT_T active site that was determined by crystallography. This step resulted in the identification of 38 compounds that showed potential docking to the FT_T active site *in silico*. In Step 3, 32 commercially available compounds out of the 38 compounds identified in Step 2, 11 herbicide control compounds and cinnamate (a compound identified as a marginal substrate in a similar enzyme family (Griffin et al., 2013)) were screened as potential substrates *in vitro* by measuring the functional response of purified FT_T protein. The list of compounds assayed, and results can be found in Table V-1.

Table V-1. *In Vitro* FT_T Enzymatic Activity Assay Compound List

Pub Chem ID[1]	Chemical Name[1]	Common Name[1]	Tag[2]	Rel. Activity[3]
5484172	(2R)-2-[4-[(6-chloro-2-quinoxalinyl)oxy]phenoxy]propanoic acid	Quizalofop-P	Herbicide	100%
185588	(2R)-2-(4-chloro-2-methylphenoxy)propanoic acid	Mecoprop-P	Herbicide	69%
91701	2-[4-[[5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid	Fluazifop	Herbicide	53%
15118048	(2R)-2-[4-(4-cyano-2-fluorophenoxy)phenoxy]propanoic acid	Cyhalofop	Herbicide	35%
8427	2-(2,4-dichlorophenoxy)propanoic acid	Dichlorprop	Herbicide	21%
1486	2-(2,4-dichlorophenoxy)acetic acid	2,4-D	Herbicide	19%
50895	2-[4-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]oxy]phenoxy]propanoic acid	Haloxyfop	Herbicide	19%
86134	2-[4-[(6-chloro-1,3-benzoxazol-2-yl)oxy]phenoxy]propanoic acid	Fenoxaprop	Herbicide	15%
7204	2-(4-chloro-2-methylphenoxy)acetic acid	MCPA	Herbicide	15%

[8] https://napralert.org/

Pub Chem ID[1]	Chemical Name[1]	Common Name[1]	Tag[2]	Rel. Activity[3]
50465	2-[(4-amino-3,5-dichloro-6-fluoro-2-pyridinyl)oxy]acetic acid	Fluroxypyr	Herbicide	8%
41428	2-[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid	Triclopyr	Herbicide	3%
4947	3,4,5-trihydroxybenzoic acid propyl ester	Propyl gallate	Endogenous	1%
637775	(E)-3-(4-hydroxy-3,5-dimethoxyphenyl)-2-propenoic acid	Sinapate	Endogenous	< 1%
7127	1,2-dimethoxy-4-prop-2-enylbenzene	Methyl Eugenol	Endogenous	< 1%
1548883	(Z)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid	Ferulic acid	Endogenous	< 1%
21685	2,6-di(butan-2-yl)phenol	N/A	Endogenous	< 1%
445858	(E)-3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid	Ferulic acid	Endogenous	< 1%
75318	2-ethoxycarbonylbenzoic acid	Monoethylphalate	Endogenous	< 1%
5281166	2-[(1R,2R)-3-oxo-2-[(Z)-pent-2-enyl]cyclopentyl]acetic acid	Jasmonic acid	Endogenous	< 1%
13988328	8-Hydroxy-2-oxo-1,2-dihydroquinoline-4-carboxylic acid	2,8-dihydroxy-4-quinolinecarboxylic acid	Endogenous	< 1%
6140	(2S)-2-amino-3-phenylpropanoic acid	Phenylalanine	Endogenous	< 1%
736186	(E)-3-(3-hydroxy-4-methoxyphenyl)-2-propenoic acid	Isoferulic acid	Endogenous	< 1%
10742	4-hydroxy-3,5-dimethoxybenzoic acid	Syringic acid	Endogenous	< 1%
802	2-(1H-indol-3-yl)acetic acid	Indole-3-acetic acid	Endogenous	< 1%
637542	(E)-3-(4-hydroxyphenyl)-2-propenoic acid	4-Hydroxycinnamic acid	Endogenous	< 1%
730037	4-(1H-indol-3-yl)-4-oxobutanoic acid	N/A	Endogenous	< 1%
325	(4-propan-2-ylphenyl)methanol	4-Isopropylbenzyl alcohol	Endogenous	< 1%
8554	benzene-1,2-dicarboxylic acid dimethyl ester	Dimethyl phthalate	Endogenous	< 1%
60961	(2R,3R,4S,5R)-2-(6-aminopurin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol	Adenosine	Endogenous	< 1%

Pub Chem ID[1]	Chemical Name[1]	Common Name[1]	Tag[2]	Rel. Activity[3]
12474015	3-ethyl-5-methoxy-1H-indole	N/A	Endogenous	< 1%
896	N-[2-(5-methoxy-1H-indol-3-yl)ethyl]acetamide	Melatonin	Endogenous	< 1%
13067	2-(1H-indol-3-yl)acetic acid ethyl ester	Ethyl 3-indoleacetate	Endogenous	< 1%
637758	(E)-3-phenyl-2-propenoic acid ethyl ester	Ethyl cinnamate	Endogenous	< 1%
6781	benzene-1,2-dicarboxylic acid diethyl ester	Diethyl phthalate	Endogenous	< 1%
17355	4-phenyl-2-butanone	Benzylacetone	Endogenous	< 1%
3314	2-methoxy-4-prop-2-enylphenol	Eugenol	Endogenous	< 1%
62428	2-(1-naphthalenyl)acetic acid ethyl ester	Ethyl 1-naphthaleneacetate	Endogenous	< 1%
6057	(2S)-2-amino-3-(4-hydroxyphenyl)propanoic acid	Tyrosine	Endogenous	< 1%
10364	2-methyl-5-propan-2-ylphenol	Carvacrol	Endogenous	< 1%
6305	(2S)-2-amino-3-(1H-indol-3-yl)propanoic acid	Tryptophan	Endogenous	< 1%
444539	(E)-3-phenylprop-2-enoic acid	Trans-cinnamate	Literature	< 1%
77021	2,7-dimethoxynaphthalene	N/A	Endogenous	< 1%
689043	(E)-3-(3,4-dihydroxyphenyl)-2-propenoic acid	Caffeic acid	Endogenous	< 1%
3080590	2-(2-oxo-1,3-dihydroindol-3-yl)acetic acid	2-oxoindole-3-acetate	Endogenous	< 1%

[1] The PubChem ID, Chemical Name, Common Name (if available) and Tag are provided (N/A is not available). https://pubchem.ncbi.nlm.nih.gov/.

[2] The column "Tag" indicates which compounds are herbicides (Herbicide), endogenous compound (Endogenous) or literature reported (Literature).

[3]The activity of triplicate assays (each with 3 replicates; n= 9) for FT_T activity reported as relative activity, which is the observed activity of the compound relative to quizalofop-P reported as a percentage. Initial velocities were first normalized in each test set against 2,4-D (positive control in each assay) and then across experiments against quizalofop-P.

Eleven herbicide controls were included in the assay. As expected, FT_T protein activity was detected in the presence of the 11 herbicide control compounds. The measured FT_T activity for all 32 endogenous plant compounds and cinnamate was ≤ 1% relative to quizalofop. These data suggest that FT_T protein is specific to substrate molecules with the following structural features: (1) existence of phenoxy group (2) presence of terminal carboxylate, and (3) available site for oxidation between the phenoxy group and terminal carboxylate. Endogenous plant small molecules with all three features did not exist in the NAPRALERT database, including the six compounds identified in the *in silico* Step 2 above that were not commercially available to test *in vitro*. Therefore, the FT_T enzyme is unlikely to metabolize endogenous small molecules in maize plants at biologically relevant activity levels.

V.D.5. FT_T Protein in MON 87429 is Not Homologous to Known Allergens or Toxins

Bioinformatics analyses were performed to assess the potential for allergenicity, toxicity, or biological activity of FT_T protein. The allergen bioinformatic results demonstrated there were no biologically relevant sequence similarities to allergens when the FT_T protein sequence was used as a query for a FASTA search of the publicly available allergen (AD_2018) database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the FT_T protein sequence and proteins in the allergen database. These data show that FT_T protein sequence lacks both structurally and immunologically relevant similarities to known allergens, gliadins, and glutenins.

FASTA bioinformatic alignment searches using the FT_T amino acid sequence were performed with a toxin database to identify possible homology with proteins that may be harmful to human and animal health. The toxin database, TOX_2018, is a subset of sequences derived from the PRT_2018 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins. The TOX_2018 database contains 28,344 sequences. The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the FT_T protein and any sequence in the TOX_2018 database.

These analyses demonstrated that FT_T protein does not share amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins which could have adverse effects on human or animal health.

V.D.6. FT_T Protein in MON 87429 is Susceptible to Degradation in *in vitro* Digestion Assay

The susceptibility to degradation by pepsin and pancreatin of MON 87429-produced FT_T was assessed using the *E. coli*-produced MON 87429 FT_T, which was shown to be equivalent to the MON 87429-produced FT_T (see Appendix D). The results indicated that the full-length, *E. coli*-produced FT_T protein is readily degraded by pepsin and pancreatin. Transient peptide fragments of ~4 kDa that were resistant (i.e., present over the course of the 60 min digestive reaction) to pepsin degradation were observed. To better understand the fate of the transiently-stable peptide fragments, sequential degradation of the FT_T protein in pepsin followed by pancreatin was

conducted. The results indicated that the transient fragments are readily degraded by sequential digestion (see Appendix N). Thus, evidence supports the conclusion that gastrointestinal digestion is sufficient to degrade the intact FT_T protein and any fragments thereof making it highly unlikely that intact or large peptide fragments of FT_T protein would be absorbed in the small intestine and have the potential to impact human or animal health.

V.D.7. FT_T Protein in MON 87429 is Not Acutely Toxic

An acute oral toxicology study with FT_T protein was conducted to provide further confirmation of the safety of this protein. There was no evidence of acute toxicity in mice when dosed orally at 2000 mg/kg body weight with FT_T protein. Based on an absence of toxicity in the acute oral toxicity study with the FT_T protein and the relatively low dietary exposure to the protein (i.e., low expression levels of FT_T protein present in MON 87429 grain (Section V.E.4 Table V-6), the risks to humans and animals following dietary exposure to the FT_T protein from consumption of food or feeds derived from MON 87429 is very low.

V.D.8. Assessment of Potential Allergenicity of the FT_T Protein

The allergenic potential of an introduced protein is assessed by comparing the biochemical characteristics of the introduced protein to biochemical characteristics of known allergens (Codex Alimentarius, 2009). Using a weight of evidence approach, a protein is not likely to be associated with allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein represents a small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence; and 4) the protein shows susceptibility to pepsin and pancreatin treatments.

The FT_T protein has been assessed for its potential allergenicity according to these safety assessment guidelines.

1) FT_T protein originates from *S. herbicidovorans*, an organism that has not been reported to be a source of known allergens.

2) FT_T protein represents a small portion of the total protein in the grain that could be consumed from MON 87429 maize due to very low expression in grain.

3) Bioinformatics analyses demonstrated that FT_T protein does not share amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes.

4) Finally, *in vitro* experiments conducted with the FT_T protein demonstrated that the protein is rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) under physiological conditions.

Taken together, these data support the conclusion that FT_T protein produced in MON 87429 does not pose a significant allergenic risk to humans or animals.

V.D.9. Human and Animal Exposure to the FT_T Protein

Based on the non-toxic mode-of-action of the FT_T protein, its susceptibility to the digestive enzymes pepsin and pancreatin, the relatively low dietary exposure to the protein (i.e., low expression levels of FT_T protein present in MON 87429 grain), and based on an absence of toxicity in a confirmatory acute oral toxicity study, it is unlikely that exposure to the FT_T protein in food and feed products derived from MON 87429 pose a risk to human and animal health.

V.E. Expression Levels of DMO, PAT, CP4 EPSPS and FT_T Proteins Produced in MON 87429

The protein expression levels determined in MON 87429 are used to assess exposure to the introduced proteins via food or feed ingestion and potential environmental exposure. The most appropriate tissues to evaluate DMO, PAT, CP4 EPSPS and FT_T protein levels are forage, leaf, root and grain tissue samples. Levels of the introduced proteins were determined in forage, leaf, root and grain tissue and were used as appropriate to evaluate potential food, feed and environmental exposures.

MON 87429 DMO, PAT, CP4 EPSPS and FT_T protein levels in various tissues of MON 87429 relevant to the characterization and risk assessment were determined using a multiplexed immunoassay. In addition, to further support the MON 87429 RHS trait mode-of-action (MOA), levels of CP4 EPSPS expression in pollen tissue was determined to demonstrate the differential expression between vegetative and pollen tissue. Tissues of MON 87429 were collected from four replicate plots planted in a randomized complete block design during the 2017 growing season at five field sites in the U.S. The field sites were representative of maize-producing regions suitable for commercial production. Forage, leaf, root, grain and pollen tissue samples were collected from plants at each replicated plot at all field sites treated with dicamba, glufosinate, quizalofop and 2,4-D herbicides (Appendix F).

V.E.1. Expression Level of DMO Protein

MON 87429 DMO protein levels were determined in forage, leaf, root and grain tissues. The results obtained from the multiplexed immunoassay are summarized in Table V-2 and the details of the materials and methods are described in Appendix F. The mean DMO protein level in MON 87429 across all sites was highest in leaf at 35 µg/g dw and lowest in root at 2.3 µg/g dw. The mean DMO protein level in MON 87429 grain was 2.4 µg/g dw.

Table V-2. Summary of DMO Protein Levels in Maize Tissues Collected from MON 87429 Produced in United States Field Trials During 2017

Tissue Type	Development Stage	Mean (SE) Range (µg/g dw)[1]	LOQ[2] (µg/g dw)
Forage	R5	21 (1.6) 9.1-32	0.14
Leaf	V2-V4	35 (2.3) 16-55	0.14
Grain	R6	2.4 (0.15) 1.3-3.6	0.14
Root	V2-V4	2.3 (0.27) 1.0-5.2	0.14

[1]Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).
[2] LOQ=limit of quantitation.

V.E.2. Expression Level of PAT Protein

PAT protein levels were determined in forage, leaf, root and grain tissues. The results obtained from the multiplexed immunoassay are summarized in Table V-3 and the details of the materials and methods are described in Appendix F. The mean PAT protein level in MON 87429 across all sites was highest in leaf at 5.8 µg/g dw and lowest in grain at 0.84 µg/g dw.

Table V-3. Summary of PAT Protein Levels in Maize Tissues Collected from MON 87429 Produced in United States Field Trials During 2017

Tissue Type	Development Stage	Mean (SE) Range (µg/g dw)[1]	LOQ[2] (µg/g dw)
Forage	R5	1.3 (0.067) 0.71-1.8	0.03
Leaf	V2-V4	5.8 (0.40) 2.9-9.8	0.03
Grain	R6	0.84 (0.066) 0.32-1.5	0.03
Root	V2-V4	2.0 (0.15) 0.40-3.1	0.03

[1]Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).
[2] LOQ=limit of quantitation.

V.E.3. Expression Level of CP4 EPSPS Protein

CP4 EPSPS protein levels were determined in forage, leaf, root and grain tissues. The results obtained from the multiplexed immunoassay are summarized in Table V-4. CP4 EPSPS protein levels were also determined in pollen to further demonstrate the differential expression between vegetative and pollen tissue resultant from the MON 87429 RHS trait mode-of-action (MOA). The results obtained from the multiplexed immunoassay are summarized in Table V-5. The details of the materials and methods for these analyses are described in Appendix F. The mean CP4 EPSPS protein level in MON 87429 across all sites was the highest in leaf at 54 µg/g dw and lowest in grain at 0.63 µg/g dw. The mean CP4 EPSPS protein level in MON 87429 pollen across all sites was below the limit of quantitation (<LOQ).

Table V-4. Summary of CP4 EPSPS Protein Levels in Maize Tissues Collected from MON 87429 Produced in United States Field Trials During 2017

Tissue Type	Development Stage	Mean (SE) Range (µg/g dw)[1]	LOQ[2] (µg/g dw)
Forage	R5	7.6 (0.50) 4.0 - 11	0.30
Leaf	V2-V4	54 (3.2) 30- 82	0.11
Grain	R6	0.63 (0.028) 0.41- 0.85	0.34
Root	V2-V4	10 (1.7) 3.4 - 29	0.11

[1]Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).
[2] LOQ=limit of quantitation.

Table V-5. Summary of CP4 EPSPS Protein Levels in Maize Pollen Tissue Collected from MON 87429 Produced in United States Field Trials During 2017

Tissue Type	Development Stage	Mean (SE) Range (µg/g dw)[1]	LOQ[2] (µg/g dw)
Pollen	R1	<LOQ	0.11

[1]Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20). Over 50% of the samples (12 of 20) were <LOQ and so < LOQ is reported here.
[2]LOQ=limit of quantitation.

V.E.4. Expression Level of FT_T Protein

FT_T protein levels were determined in forage, leaf, root and grain tissues. The results obtained from the multiplexed immunoassay are summarized in Table V-6 and the details of the materials and methods are described in Appendix F. The mean FT_T protein level in MON 87429 across all sites was the highest in leaf at 440 µg/g dw and lowest in root at 41 µg/g dw. The mean FT_T protein level in MON 87429 grain was 47 µg/g dw.

Table V-6. Summary of FT_T Protein Levels in Maize Tissues Collected from MON 87429 Produced in United States Field Trials During 2017

Tissue Type	Development Stage	Mean (SE) Range (µg/g dw)[1]	LOQ[2] (µg/g dw)
Forage	R5	97 (5.2) 56 - 140	0.036
Leaf	V2-V4	440 (25) 210 - 670	0.036
Grain	R6	47 (3.6) 19 - 79	0.036
Root	V2-V4	41 (4.1) 7.2 - 82	0.036

[1]Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).
[2] LOQ=limit of quantitation.

V.F. MON 87429 DMO, PAT, CP4 EPSPS and FT_T Proteins Characterization and Safety Conclusion

MON 87429 DMO is an enzyme that catalyzes the demethylation of dicamba to the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) and formaldehyde. DMO proteins have been previously characterized, and the safety of these proteins and crops expressing these proteins has been well established. Expression studies using immunoassay demonstrated that DMO was expressed at low levels in grain. Taken together, the evidence indicates that the consumption of the DMO protein from MON 87429 or its progeny is safe for humans and animals.

PAT protein is an acetyltransferase that catalyzes the acetylation of the herbicide glufosinate. PAT proteins, including the PAT protein isolated from MON 87429, have been previously characterized, and the safety of these proteins and crops expressing these proteins has been well established. Expression studies using immunoassay demonstrated

that PAT was expressed at low levels in grain. Taken together, the evidence indicates that the consumption of the PAT protein from MON 87429 or its progeny is safe for humans and animals.

Members of the EPSPS protein family, including CP4 EPSPS, are enzymes which catalyze a key step in aromatic amino acid biosynthesis and catalyzes the reaction where the enolpyruvyl group from phosphoenol pyruvate (PEP) is transferred to the 5-hydroxyl of shikimate-3-phosphate to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate. CP4 EPSPS proteins, including the CP4 EPSPS protein isolation from MON 87429, have been previously characterized, and the safety of these proteins and crops expressing these proteins has been well established. Expression studies using immunoassay demonstrated that CP4 EPSPS was expressed at low levels in grain. Taken together, the evidence indicates that the consumption of the CP4 EPSPS protein from MON 87429 or its progeny is safe for humans and animals.

The FT_T protein is an alpha-ketoglutarate-dependent non-heme iron dioxygenase that catalyzes a dioxygenase reaction in the presence of alpha-ketoglutarate (αKG) and oxygen by incorporating oxygen into quizalofop, thus degrading it into the herbicidally-inactive quizalofop phenol and pyruvate, while maintaining specificity for its substrate. The physicochemical characteristics of the MON 87429-produced FT_T protein were determined and equivalence between MON 87429-produced FT_T and *E. coli*-produced FT_T proteins was demonstrated. This equivalence justifies the use of the *E. coli*-produced FT_T as a test substance in the protein safety studies (acute oral toxicity and digestibility). Expression studies using immunoassay demonstrated that FT_T was expressed at low levels in grain. The FT_T protein lacks structural similarity to allergens, toxins or other proteins known to produce adverse effects in mammals. In addition, the ubiquitous presence of *Sphingobium* species in the environment has resulted in widespread human and animal exposure and is not commonly known for allergenicity and human or animal pathogenicity. The FT_T protein is rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) and demonstrated no acute oral toxicity in mice at the high dose tested. Based on the above information, the consumption of the FT_T protein in foods derived from MON 87429 or its progeny is considered safe for humans and animals.

The protein safety data presented herein support the conclusion that food and feed products containing MON 87429 or derived from MON 87429 are as safe for human and animal consumption as maize currently on the market and expression of the DMO, PAT, CP4 EPSPS and FT_T proteins in MON 87429 does not impact the weediness or plant pest risk of MON 87429 maize.

VI. COMPOSITIONAL ASSESSMENT OF MON 87429

Food and feed safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius, 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Maize is not known to have any endogenous toxicants or antinutrients associated with overall plant pest potential (OECD, 2002b). For MON 87429, the introduced proteins, DMO, PAT, CP4 EPSPS and FT_T, confer herbicide tolerance and lack catalytic activity that is intended to or expected to affect the plant's metabolism. Given the nature of these introduced traits and the overall lack of meaningful unintended compositional characteristics observed for biotechnology-derived products characterized to date (Herman and Price, 2013; Venkatesh et al., 2015), compositional changes that would affect the levels of components in MON 87429 maize were not expected.

Monsanto is currently in consultation with the Food and Drug Administration following their policy, "Foods Derived from New Plant Varieties," on the food and feed safety of MON 87429 maize (Submitted 05-Feb-2019). Samples were collected from 5 field sites grown in 2017 that were representative of U.S. maize growing regions. Composition data for 25 components including major nutrients in grain (protein, amino acids, total fat, linoleic acid, carbohydrates, acid detergent fiber, neutral detergent fiber and ash), major nutrients in forage (protein, total fat, carbohydrates, acid detergent fiber, neutral detergent fiber and ash) and anti-nutrients in grain (phytic acid and raffinose) were submitted to FDA as part of the voluntary food/feed safety and nutritional assessment for MON 87429 maize. The results of the compositional assessment found that there were no biologically meaningful differences between MON 87429 and conventional control and support the conclusion that MON 87429 maize is compositionally equivalent to the conventional control.

This section provides analyses of concentrations of key nutrients and anti-nutrients in grain and forage of MON 87429 compared to that of a conventional control maize hybrid grown and harvested under similar conditions. The production of materials for compositional analyses used a sufficient variety of field trial sites, reflecting a range of environmental conditions under which MON 87429 is expected to be grown and robust field designs (randomized complete block design with four replicates). Samples were subjected to sensitive analytical methods that allow quantitative and accurate measurements of key components. See Appendix M for details on composition methods.

VI.A. Compositional Equivalence of MON 87429 Grain and Forage to Conventional Maize

Grain and forage samples were harvested from MON 87429 and a conventional control grown at five sites in the United States during 2017. The field sites were planted in a randomized complete block design with four replicates per site. MON 87429 and the conventional control were grown under agronomic field conditions typical for each of the different growing regions. MON 87429 plots were treated with dicamba, glufosinate,

quizalofop and 2,4-D to generate samples under conditions of the intended use of the product.

The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients and anti-nutrients in grain and forage of MON 87429 and the conventional control.

Compositional analyses of grain and forage samples are reported for a subset of components listed in the maize OECD consensus document (OECD, 2002b). Harvested grain samples were assessed for moisture and levels of key nutrients including proximates (protein, total fat and ash), essential amino acids (10 components), linoleic acid (essential fatty acid), carbohydrates by calculation and fiber (acid detergent fiber (ADF) and neutral detergent fiber (NDF)). Grain samples were also assessed for levels of antinutrients (phytic acid and raffinose). Harvested forage samples were assessed for moisture and levels of nutrients including proximates (protein, total fat and ash), carbohydrates by calculation and fiber (ADF and NDF). In all, 27 different components were analyzed. Moisture values for grain and forage were measured to enable the conversion of components from fresh to dry weight but were not statistically analyzed. Therefore, 25 components were statistically analyzed.

The statistical comparison of MON 87429 and the conventional control was based on compositional data combined across all five field sites. Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). A statistically significant difference between MON 87429 and the conventional control does not necessarily imply biological relevance from a food and feed safety perspective. Therefore, any statistically significant differences observed between MON 87429 and the conventional control were evaluated further to determine whether the detected difference indicated a biologically relevant compositional change or supported a conclusion of compositional equivalence, as follows:

Step 1 – Determination of the Magnitude of Difference between Test (MON 87429) and Conventional Control Means

The difference in means between MON 87429 and the conventional control was determined for use in subsequent steps.

Step 2 – Assessment of the Difference in the Context of Natural Variation within the Conventional Control across Multiple Sites

The difference between MON 87429 and the conventional control was evaluated in the context of variation within the conventional control germplasm grown across multiple sites (i.e., variation due to environmental influence) by determining the range of replicate values for the conventional control (range value = maximum value minus the minimum value). A mean difference less than the variability seen due to natural environmental variation within the single, closely related germplasm is typically not a food or feed safety concern (Venkatesh et al., 2014).

Step 3 – Assessment of the Difference in the Context of Natural Variation Due to Multiple Sources

The relative impact of MON 87429 on composition was evaluated in the context of sources of natural variation such as environmental and germplasm influences. This assessment determined whether the component mean value of MON 87429 was within the natural variability defined by the literature values or the ILSI Crop Composition Database (ILSI-CCDB) values. This natural variability is important in assessing the biological relevance to food and feed safety of statistically significant differences in composition between MON 87429 and the conventional control.

These evaluations of natural variation are important as crop composition is known to be greatly influenced by environment and variety (Harrigan et al., 2010). Although used in the comparative assessment process, detection of statistically significant differences between MON 87429 and the conventional control mean values does not necessarily imply a meaningful contribution by MON 87429 to compositional variability. Only if the impact of MON 87429 on levels of components is large relative to natural variation inherent to conventional maize would the difference in composition be potentially meaningful from a food and feed safety and nutritional perspective. Differences between MON 87429 and the conventional control that are within the observed natural variation for maize would support a conclusion of compositional equivalence.

Compositional Equivalence of MON 8729 Grain and Forage to that of Conventional Maize

There were no statistically significant differences ($p<0.05$) for 23 of the 25 components analyzed from MON 87429 grain and forage (Table VI-1–Table VI-4). There were two components (total fat and linoleic acid in grain) that showed a statistically significant difference ($p<0.05$) between MON 87429 and the conventional control (Table VI-1). For total fat, the mean value was 3.76 % dw for MON 87429 and 3.88 % dw for the conventional control, a difference of -0.15 % dw. For linoleic acid, the mean value was 55.53 % Total Fatty Acid (FA) for MON 87429 and 55.21 % Total FA for the conventional control, a difference of 0.32 % Total FA. For these components, the mean difference between MON 87429 and the conventional control was less than the conventional control range values and the MON 87429 mean component values were also within the range of values observed in the literature and/or the ILSI-CCDB values (Table VI-5).

These data indicated that the statistically significant differences observed in total fat and linoleic acid in grain were not biologically meaningful from a food and feed safety perspective. These results support the conclusion that MON 87429 was not a major contributor to variation in component levels in maize grain or forage and confirmed the compositional equivalence of MON 87429 to the conventional control in levels of key nutrients and anti-nutrients in grain and forage.

Table VI-1. Summary of Maize Grain Proximates, Linoleic Acid, Carbohydrates by Calculation and Fiber for MON 87429 and Conventional Control

Component	MON 87429 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Difference (Test minus Control) Mean (S.E.)	p-Value
Protein (% dw)[3]	9.20 (0.30) 7.95 - 10.99	9.35 (0.30) 8.04 - 11.01	2.96	-0.15 (0.14)	0.289
Total fat (% dw)	3.76 (0.050) 3.46 - 4.01	3.88 (0.050) 3.58 - 4.16	0.57	-0.12 (0.045)	0.049
Linoleic acid (% Total FA)[4]	55.53 (0.63) 53.61 - 57.87	55.21 (0.63) 53.28 - 57.38	4.10	0.32 (0.12)	0.018
Ash (% dw)	1.15 (0.057) 0.97 - 1.34	1.15 (0.057) 0.91 - 1.41	0.50	-0.0013 (0.024)	0.958
Carbohydrates by calculation (% dw)	85.87 (0.38) 83.97 - 87.27	85.61 (0.38) 83.62 - 87.34	3.73	0.25 (0.15)	0.104
ADF (% dw)	2.63 (0.078) 1.94 - 3.67	2.53 (0.078) 2.28 - 3.03	0.74	0.098 (0.11)	0.378

Table VI-1. Summary of Maize Grain Proximates, Linoleic Acid, Carbohydrates by Calculation and Fiber for MON 87429 and Conventional Control (continued)

Component	MON 87429 Mean (S.E.)[1] Range	Control Mean (S.E.) Range	Control Range Value[2]	Difference (Test minus Control) Mean (S.E.)	p-Value
NDF (% dw)[3]	7.82 (0.16) 6.97 - 8.63	8.05 (0.16) 6.44 - 9.80	3.36	-0.22 (0.23)	0.339

[1]Mean (S.E.) = least-square mean (standard error)
[2]Maximum value minus minimum value for the control maize hybrid
[3]dw=dry weight
[4]FA=Fatty Acid

Table VI-2. Summary of Maize Grain Essential Amino Acids for MON 87429 and Conventional Control

Component (% dw)[1]	MON 87429 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Arginine	0.45 (0.014) 0.40 - 0.54	0.45 (0.014) 0.38 - 0.51	0.13	0.0047 (0.0068)	0.498
Histidine	0.26 (0.0091) 0.22 - 0.31	0.25 (0.0091) 0.22 - 0.30	0.085	0.0018 (0.0053)	0.739
Isoleucine	0.34 (0.013) 0.30 - 0.43	0.34 (0.013) 0.29 - 0.41	0.12	0.0013 (0.0069)	0.850
Leucine	1.20 (0.054) 0.98 - 1.56	1.22 (0.054) 1.00 - 1.51	0.52	-0.012 (0.027)	0.672
Lysine	0.26 (0.0077) 0.22 - 0.35	0.26 (0.0077) 0.21 - 0.28	0.067	0.0062 (0.0084)	0.499
Methionine	0.20 (0.0067) 0.17 - 0.25	0.20 (0.0067) 0.17 - 0.24	0.065	-0.0040 (0.0026)	0.135

Table VI-2. Summary of Maize Grain Essential Amino Acids for MON 87429 and Conventional Control (continued)

Component (% dw)[1]	MON 87429 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Difference (Test minus Control) Mean (S.E.)	Difference (Test minus Control) p-Value
Phenylalanine	0.48 (0.020) 0.40 - 0.59	0.48 (0.020) 0.40 - 0.60	0.20	0.0022 (0.010)	0.845
Threonine	0.34 (0.0097) 0.30 - 0.42	0.34 (0.0097) 0.30 - 0.39	0.096	0.0010 (0.0056)	0.853
Tryptophan	0.074 (0.0016) 0.065 - 0.080	0.075 (0.0016) 0.066 - 0.083	0.017	-0.0012 (0.0016)	0.479
Valine	0.44 (0.015) 0.39 - 0.55	0.44 (0.015) 0.38 - 0.52	0.14	0.0032 (0.0072)	0.656

[1]dw=dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Maximum value minus minimum value for the control maize hybrid

Table VI-3. Summary of Maize Grain Anti-Nutrients for MON 87429 and Conventional Control

				Difference (Test minus Control)	
Component (% dw)[1]	MON 87429 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Mean (S.E.)	p-Value
Phytic acid	0.64 (0.060) 0.39 - 0.91	0.67 (0.060) 0.44 - 0.91	0.47	-0.033 (0.032)	0.355
Raffinose	0.15 (0.020) 0.097 - 0.23	0.16 (0.020) 0.11 - 0.26	0.15	-0.010 (0.0056)	0.083

[1]dw=dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Maximum value minus minimum value for the control maize hybrid

Table VI-4. Summary of Maize Forage Proximates, Carbohydrates by Calculation and Fiber for MON 87429 and Conventional Control

				Difference (Test minus Control)	
Component (% dw)[1]	MON 87429 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Control Range Value[3]	Mean (S.E.)	p-Value
Protein	7.10 (0.20) 4.57 - 8.93	7.00 (0.20) 6.03 - 8.21	2.18	0.10 (0.22)	0.654
Total fat	2.93 (0.14) 2.20 - 3.54	2.81 (0.14) 1.58 - 3.41	1.83	0.12 (0.17)	0.507
Carbohydrates by calculation	86.32 (0.24) 84.46 - 89.56	86.51 (0.24) 85.04 - 88.11	3.07	-0.19 (0.33)	0.594
ADF	20.59 (0.92) 15.74 - 24.53	21.06 (0.92) 14.63 - 28.26	13.63	-0.47 (0.79)	0.562
NDF	33.63 (1.20) 26.99 - 44.93	34.76 (1.20) 26.95 - 42.49	15.54	-1.13 (1.39)	0.422
Ash	3.65 (0.22) 3.06 - 4.58	3.68 (0.22) 2.80 - 4.72	1.92	-0.034 (0.10)	0.743

[1]dw=dry weight
[2]Mean (S.E.) = least-square mean (standard error)
[3]Maximum value minus minimum value for the control maize hybrid

Table VI-5. Literature and ILSI-CCDB Database Ranges for Components in Maize Grain and Forage

Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Grain Nutrients		
Proximates		
protein (% dw)	8.27-13.33[a]; 9.17-12.19[b]	5.72-17.26
total fat (% dw)	2.95-4.40[a]; 3.18-4.23[b]	1.363-7.830
ash (% dw)	1.17-2.01[a]; 1.27-1.63[b]	0.616-6.282
Amino Acids		
arginine (% dw)	0.34-0.52[a]; 0.34-0.50[b]	0.12-0.71
histidine (% dw)	0.25-0.37[a]; 0.27-0.34[b]	0.14-0.46
isoleucine (% dw)	0.30-0.48[a]; 0.32-0.44[b]	0.18-0.69
leucine (% dw)	1.02-1.87[a]; 1.13-1.65[b]	0.64-2.49
lysine (% dw)	0.26-0.33[a]; 0.28-0.31[b]	0.129-0.668
methionine (% dw)	0.17-0.26[a]; 0.16-0.30[b]	0.11-0.47
phenylalanine (% dw)	0.43-0.72[a]; 0.45-0.63[b]	0.24-0.93
threonine (% dw)	0.29-0.45[a]; 0.31-0.39[b]	0.22-0.67
tryptophan (% dw)	0.047-0.085[a]; 0.042-0.07[b]	0.027-0.215
valine (% dw)	0.42-0.62[a]; 0.45-0.58[b]	0.27-0.86
Fatty Acids		
linoleic acid (% Total FA)	49.31-64.70[a]; 56.51-65.65[b]	34.27-67.68
Carbohydrates By Calculation		
carbohydrates by calculation (% dw)	81.31-87.06[a]; 82.10-85.98[b]	77.4-89.7
Fiber		
ADF (% dw)	1.82-4.48[a]; 1.83-3.39[b]	1.41-11.34
NDF (% dw)	6.51-12.28[a]; 6.08-10.36[b]	4.28-22.64
Anti-Nutrients		
phytic acid (% dw)	0.69-1.09[a]; 0.60-0.94[b]	0.111-1.940
raffinose (% dw)	0.079-0.22[a]; 0.061-0.15[b]	0.020-0.466
Forage Nutrients		
Proximates		
protein (% dw)	5.80-10.24[a]; 5.56-9.14[b]	3.14-16.32
total fat (% dw)	1.28-3.62[a]; 0.20-1.76[b]	0.296-6.755
ash (% dw)	2.67-8.01[a]; 4.59-6.9[b]	0.66-13.20
Carbohydrates By Calculation		
carbohydrates by calculation (% dw)	81.88-89.26[a]; 84.11-87.54[b]	73.3-92.9
Fiber		
ADF (% dw)	19.11-30.49[a]; 20.73-33.39[b]	9.90-47.39
NDF (% dw)	27.73-49.62[a]; 31.81-50.61[b]	20.29-67.80

[1]dw=dry weight; FA=Fatty Acid.
[2]Literature range references: [a](Harrigan et al., 2009) (see U.S. Field data);[b](Harrigan et al., 2009) (see Chile field data).
[3]ILSI range is from ILSI Crop Composition Database, 2016 (Accessed February 21, 2017).

VII. PHENOTYPIC, AGRONOMIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

This section provides a comparative assessment of the phenotypic, agronomic, and environmental interactions characteristics of MON 87429 compared to the conventional control. The data support a conclusion that MON 87429 is unlikely to pose a plant pest risk. This conclusion is based on the results of multiple evaluations including field and laboratory assessments. MON 87429 has been planted in the U.S. under permit/notification since 2014 (Appendix A).

Phenotypic, agronomic, and environmental interactions characteristics of MON 87429 were evaluated in a comparative manner to assess plant pest potential. These assessments included seed germination and dormancy characteristics and pollen characteristics in the laboratory as well as phenotypic and agronomic characteristics and plant responses to abiotic stressors, diseases, and arthropod pests in the field. Results from these assessments demonstrate that MON 87429 does not possess 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests; or 3) characteristics that would confer a plant pest risk compared to conventional maize.

VII.A. Characteristics Measured for Assessment

In the phenotypic, agronomic, and environmental interactions assessment of MON 87429, data were collected to evaluate altered plant pest potential. A detailed description of the regulated article phenotype is requested as part of the petition for determination of nonregulated status in 7 CFR § 340.6 including differences from the unmodified recipient organism that would "substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived." As part of the characterization of MON 87429, data were collected to provide a detailed description of the phenotypic, agronomic, and environmental interactions characteristics of MON 87429. A subset of these data represents specific characteristics that are typically associated with altered plant pest potential (e.g., seed dormancy, lodging, seed loss, and environmental interactions data).

The plant characterization of MON 87429 encompassed five general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development (including pollen characteristics); 4) lodging and seed retention on the plant; and 5) plant responses to abiotic stressors, diseases, and arthropod pests. An overview of the characteristics assessed is presented in Table VII-1.

The phenotypic, agronomic, and environmental interactions data were evaluated from a basis of familiarity (OECD, 1993) and were comprised of a combination of field and laboratory studies conducted by scientists who are familiar with the production and evaluation of maize. In each of these assessments, MON 87429 was compared to an appropriate conventional control that had a genetic background similar to MON 87429 but did not possess the inserted traits. In addition, multiple commercial maize hybrids developed through conventional breeding and selection (see Appendices H-J and Tables

H-1, I-1, and J-1) were included to provide a range of comparative values for each characteristic that are representative of the variability in existing commercial maize hybrids. Data collected for the various characteristics from the commercial reference maize hybrids provide context for interpreting experimental results.

Table VII-1. Phenotypic, Agronomic, and Environmental Interactions Characteristics Evaluated in U.S. Field Trials and Laboratory Studies

Data category	Characteristic measured (section where discussed)	Evaluation timing (setting of evaluation)	Evaluation description (measurement endpoints)
Germination, dormancy, and emergence	Germinated (VII.C.1)	Day 4 and 7 at OT[1] Day 4, 7, and 11 at SOT[1] (Laboratory)	Percentage of seed with a radicle protruding through the seed coat and greater than 1 mm in length
	Dead (VII.C.1)	Day 4 and 7 at OT Day 4, 7, and 11 at SOT (Laboratory)	Percentage of seed that had visibly deteriorated and become soft to the touch (also included non-viable hard and non-viable firm-swollen seed)
	Viable firm-swollen (VII.C.1)	Day 7 at OT Day 11 at SOT (Laboratory)	Percentage of seed that imbibed water and were firm to the touch but lacked any evidence of growth (viability determined by a tetrazolium test)
	Viable hard (VII.C.1)	Day 7 at OT Day 11 at SOT (Laboratory)	Percentage of seed that did not imbibe water and remained hard to the touch (viability determined by a tetrazolium test)
	Early stand count (VII.C.2.1)	V2 – V5 growth stage (Field)	Number of plants per m^2
Vegetative growth	Plant height (VII.C.2.1)	R1 – R6 (Field)	Distance from the soil level to the flag leaf collar
	Days to maturity (VII.C.2.1)	R6 (Field)	Number of days from planting to kernel black layer
	Final stand count (VII.C.2.1)	Pre-harvest (Field)	Number of plants per m^2

[1] Optimum temperature OT = 20/30°C, Suboptimum temperature SOT = 10°C for seven days followed by 25°C for four days.

Table VII-I. Phenotypic, Agronomic, and Environmental Interactions Characteristics Evaluated in U.S. Field Trials and Laboratory Studies (continued)

Data category	Characteristic measured (section where discussed)	Evaluation timing (setting of evaluation)	Evaluation description (measurement endpoints)
Reproductive development	Days to flowering (VII.C.2.1)	R1 (Field)	Days from planting until 50% of the plants have begun to shed pollen
	Pollen viability (VII.C.3)	R1 (Laboratory)	Percentage of viable pollen based on pollen grain staining characteristics
	Pollen diameter (VII.C.3)	R1 (Laboratory)	Diameter of representative pollen grains
	Grain moisture (VII.C.2.1)	Harvest (Field)	Percentage moisture of harvested grain
	Seed weight (VII.C.2.1)	Harvest (Field)	Mass of 100 mature seeds, adjusted to 15.5% moisture content
	Yield (VII.C.2.1)	Harvest (Field)	Mass of harvested grain per hectare, adjusted to 15.5% moisture content
Lodging and seed retention	Lodging (VII.C.2.1)	Pre-harvest (Field)	Percentage of plants leaning >45° from vertical or broken below the ear
	Seed loss (VII.C.2.1)	Pre-harvest (Field)	Number of ears completely detached from plants in two rows
Environmental interactions	Abiotic stress response (VII.C.2.2)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Disease damage (VII.C.2.2)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)
	Arthropod damage (VII.C.2.2)	Four times during growing season (Field)	Qualitative assessment of each plot, with categorical scale of increasing severity (none, slight, moderate, severe)

[1] Optimum temperature OT = 20/30°C, Suboptimum temperature SOT = 10°C for seven days followed by 25°C for four days.

VII.B. Data Interpretation

Plant pest risk assessments for biotechnology-derived crops are comparative assessments and are considered from a basis of familiarity. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a well-characterized conventional maize whose biological properties and plant pest potential are well-known. Familiarity considers the biology of the crop, the introduced traits, the receiving environment and the interaction of these factors, and provides a basis for comparative environmental risk assessment between a biotechnology-derived plant and its conventional counterpart.

Expert knowledge and experience with conventionally bred maize was the basis for selecting appropriate endpoints and estimating the range of responses that would be considered typical for maize. As such, MON 87429 was compared to the conventional control in the assessment of phenotypic, agronomic, and environmental interactions characteristics. Based on all of the data collected, an assessment was made to determine if MON 87429 could be expected to pose an increased plant pest risk compared to conventional maize.

VII.B.1. Interpretation of Phenotypic and Agronomic Data

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased weediness or plant pest risk. Under the framework of familiarity, characteristics for which no differences are detected support a conclusion of no increased weediness or plant pest risk of the biotechnology-derived crop compared to the conventional crop. Characteristics for which statistically significant differences are detected are considered in a step-wise method (Figure VII-1) or in a similar fashion. All detected differences for a characteristic are considered in the context of whether or not the difference would increase the crop's pest/weed potential. Ultimately, a weight of evidence approach considering all characteristics and data is used for the overall risk assessment of differences and their significance. Figure VII-1 illustrates the stepwise assessment process employed.

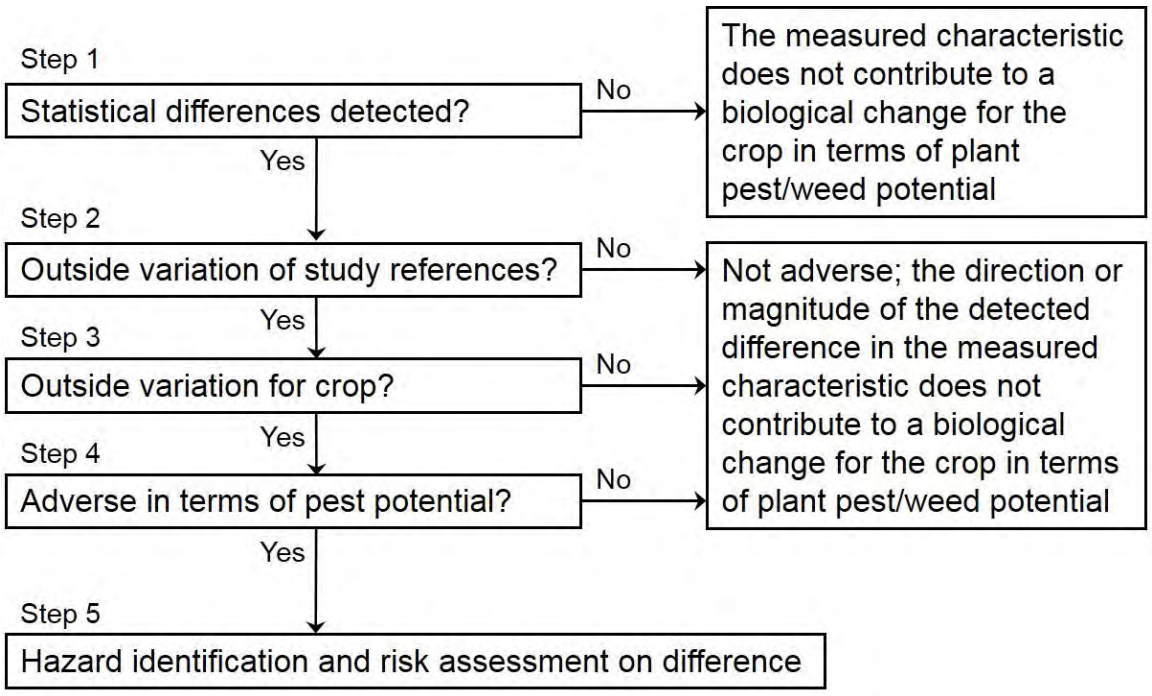


Figure VII-1. Interpretation of Statistical Differences

Step 1 – Evaluate Detected Statistically Significant Differences

Data on each measured characteristic are statistically analyzed. A combined-site analysis is used for multi-site data. All statistically significant differences are evaluated and considered in the context of a change in weediness or plant pest risk. Any difference detected is further assessed.

Step 2 – Evaluate Differences in the Context of Commercial Reference Materials Included in the Study

If a difference for a characteristic is detected then the mean value of the biotechnology-derived crop for the characteristic is assessed relative to the range of variation of the commercial reference materials included in the study (e.g., reference range).

Step 3 – Evaluate Differences in the Context of the Crop

If the mean value of the characteristics for a biotechnology-derived crop is outside the variation of the commercial reference materials included in the study, the mean value of the biotechnology-derived crop is assessed relative to known values common for the crop (e.g., published values).

Step 4 – Relevance of Difference to Weediness or plant pest risk

If the mean value of the characteristics for a biotechnology-derived crop is outside the range of values common for the crop, the difference is then assessed for whether or not it is meaningful in terms of weediness or plant pest risk.

Step 5 – Conduct Risk Assessment on Identified Hazard

If an adverse effect (hazard) is identified, a risk assessment on the difference is conducted. The risk assessment considers contributions to enhanced weediness or plant pest risk of the crop itself, the impact of differences detected in other measured characteristics, and potential for and effects of trait introgression into any populations growing outside of cultivated environments or into a sexually compatible species.

VII.B.2. Interpretation of Environmental Interactions Data

The environmental interactions data consisting of plant responses to abiotic stressors, diseases, and arthropod pests are categorical and were considered different in susceptibility or tolerance if the range of injury symptoms did not overlap between the biotechnology-derived crop and the conventional control across all four replications within an observation at a site.

Observations for which no differences are detected support a conclusion of no increased weediness or plant pest risk. Observations for which differences are detected are not considered to increase weediness or plant pest risk if the biotechnology-derived crop stressor responses and damage ratings are within the reference range or are not consistently observed in multiple environments in which the same stressor occurred.

VII.C. Comparative Assessments of the Phenotypic, Agronomic, and Environmental Interactions Characteristics of MON 87429

This section provides the results of comparative assessments conducted in replicated laboratory and/or multi-site field experiments to provide a detailed phenotypic, agronomic, and environmental interactions description of MON 87429. The characteristics for MON 87429 evaluated in these assessments included: seed germination and dormancy characteristics (Section VII.C.1), plant phenotypic, agronomic, and environmental interactions observations under field conditions (Section VII.C.2), and pollen characteristics (Section VII.C.3). Additional details for each assessment are provided in Appendix H, Appendix I, and Appendix J.

VII.C.1. Seed Germination and Dormancy Characteristics

USDA-APHIS considers the potential for weediness to constitute a plant pest factor (7 CFR § 340.6). Seed germination and dormancy mechanisms vary with species and their genetic basis tends to be complex. Seed dormancy (e.g., hard seed) is an important characteristic that is often associated with plants that are considered weeds (Anderson, 1996; Lingenfelter and Hartwig, 2007). Information on germination and dormancy characteristics is therefore useful when assessing a plant for increased weediness potential. To assess germination characteristics, standardized germination assays are available and routinely used. The Association of Official Seed Analysts (AOSA), an internationally recognized seed testing organization, recommends a temperature regime of alternating 20°C and 30°C for testing the germination and dormancy characteristics of maize seed (AOSA, 2017a; b). The AOSA further recognizes a temperature regime of constant 10°C for seven days followed by 25°C for four days for cold testing of maize (AOSA, 2009).

A comparative assessment of seed germination and dormancy characteristics was conducted for MON 87429 and the conventional control. The seed lots for MON 87429 and the conventional control were harvested from one 2017 field production site in Kihei, Hawaii. Four reference maize hybrids were obtained from commercial sources. MON 87429 was compared to the conventional control for percentages of germinated, dead, viable firm-swollen, and viable hard seed using two temperature regimes: optimum (alternating 20°C and 30°C) and suboptimum (AOSA cold test: constant 10°C for seven days followed by 25°C for four days). The assay for each temperature regime was conducted using a randomized complete block design with four replications. Descriptions of the evaluated germination and dormancy characteristics and the timing of the evaluations are listed in Table VII-1. The materials and experimental methods are further discussed in Appendix H

In the analyses of the dormancy and germination data, no statistically significant differences ($\alpha = 0.05$) were detected between MON 87429 and the conventional control in either the optimum (20/30°C) or suboptimum (10/25°C) temperature regimes for any of the evaluated characteristics, including viable hard seed (Table VII-2). These results support the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize.

Table VII-2. Germination and Dormancy Characteristics of MON 87429 and the Conventional Control

Temperature Regime (°C)[1]	Characteristic[2]	Mean % (SE)[3]		Reference Range
		MON 87429	Control	
Optimum	Germinated	93.0 (1.47)	96.3 (0.48)	99.0 – 100.0
	Dead	7.0 (1.47)	3.8 (0.48)	0.0 – 1.0
	Viable firm-swollen	0.0 (0.00)	0.0 (0.00)	0.0 – 0.0
	Viable hard	0.0 (0.00)	0.0 (0.00)	0.0 – 0.0
Suboptimum	Germinated	94.5 (1.26)	96.3 (1.25)	99.3 – 100.0
	Dead	5.5 (1.26)	3.8 (1.25)	0.0 – 0.8
	Viable firm-swollen	0.0 (0.00)	0.0 (0.00)	0.0 – 0.0
	Viable hard	0.0 (0.00)	0.0 (0.00)	0.0 – 0.0

Notes: No statistically significant differences were detected between MON 87429 and the conventional control (α =0.05).

[1] The optimum temperature regime was 20/30ºC for seven days; The suboptimum temperature regime was 10ºC for seven days followed by 25ºC for four days.

[2] Statistical comparisons were performed using ANOVA (optimum temperature regime) or Fisher's Exact Test (suboptimum temperature regime). No statistical comparisons were made if MON 87429 and control values were 0.

[3] N= 4 for means. S.E. = standard error.

VII.C.2. Field Phenotypic, Agronomic and Environmental Interactions Characteristics

Phenotypic and agronomic characteristics were evaluated under field conditions as part of the plant characterization assessment of MON 87429. These data were developed to provide USDA-APHIS with a detailed description of MON 87429 relative to the conventional control and reference maize hybrids. According to 7 CFR § 340.6, as part of the petition to seek deregulation, a petitioner must submit "a detailed description of the phenotype of the regulated article." This information is being provided to assess whether there are phenotypic differences between MON 87429 and the conventional control that may impact its weediness or plant pest risk. Specific characteristics that are typically associated with weediness (e.g., lodging and seed loss) were used to assess whether there is a potential increase in weediness of MON 87429 compared to conventional maize.

USDA-APHIS considers the environmental interaction of the biotechnology-derived crop compared to its conventional control to determine the potential for increased plant pest characteristics. Evaluations of environmental interactions were conducted as part of the plant characterization for MON 87429. In the 2017 U.S. field trials conducted to evaluate the phenotypic and agronomic characteristics of MON 87429, data were also collected on plant responses to abiotic stressors, diseases, and arthropod pests.

Data were collected from eight 2017 field sites within maize production regions of the U.S. (Table VII-3). The test material MON 87429, the conventional control, and four commercial reference hybrids were planted at each site in a randomized complete block design with four replications. Sixteen unique references were included among the sites. Additional details on the materials and methods are presented in Appendix I.

VII.C.2.1. Phenotypic and Agronomic Assessments

MON 87429 was compared to the conventional control in a combined-site analysis for nine phenotypic and agronomic characteristics: early stand count, days to flowering, plant height, days to maturity, lodging, final stand count, moisture, seed weight, and yield. Descriptive statistics are provided for an additional characteristic, seed loss, that had insufficient variability for formal statistical analysis. Descriptions of the evaluated phenotypic and agronomic characteristics and the timing of the evaluations are listed in Table VII-1. The materials and methods are further discussed in Appendix I.

The means for MON 87429 and the conventional control for seed loss, which was excluded from formal analysis, were low (0.2 and 0.1 ears/two rows, respectively) and the mean for MON 87429 was within the reference range. These results suggest that this characteristic does not contribute to a biological change for MON 87429 in terms of plant pest potential.

In a combined-site analysis of phenotypic and agronomic characteristics of MON 87429 compared to the conventional control, no statistically significant differences ($\alpha = 0.05$) were detected between MON 87429 and the conventional control for any of the analyzed characteristics (Table VII-4). These results, together with those for seed loss, support the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize.

Table VII-3. Phenotypic and agronomic and Environmental Interactions Sites for MON 87429 during 2017

Site Code	County, State
IAAU	Audubon, Iowa
IARL	Jefferson, Iowa
ILCX	Vermilion, Illinois
ILMN	Warren, Illinois
INSH	Boone, Indiana
NESW	Seward, Nebraska
NEYO	York, Nebraska
OHTR	Miami, Ohio

Table VII-4. Combined-Site Analysis of Phenotypic and Agronomic Characteristics of MON 87429 Compared to the Conventional Control in 2017 U.S. Field Trials

Characteristic (units)	Mean (S.E.)[1]		Reference Range[2]
	MON 87429	Control	
Early stand count (plants/m^2)	9.0 (0.08)	9.1 (0.07)	8.5 – 9.5
Days to flowering	63.6 (0.86)	63.5 (0.81)	57.3 – 69.0
Plant height (cm)	241.2 (3.41)	243.3 (3.06)	181.5 – 251.1
Days to maturity	131.7 (1.34)	130.5 (1.61)	118.8 – 139.6
Lodging (%)	8.3 (2.03)	10.3 (3.60)	0.0 – 16.2
Seed loss (ears/two rows)	0.2 (0.13)†	0.1 (0.09)	0.0 – 0.2
Final stand count (plants/m^2)	8.4 (0.11)	8.4 (0.12)	7.8 – 9.3
Moisture (%)	18.7 (0.43)	18.6 (0.45)	15.1 – 23.6
Seed weight (g)	37.2 (0.74)	36.8 (0.75)	31.1 – 43.1
Yield (t/ha)	15.1 (0.32)	14.8 (0.34)	13.1 – 18.9

Notes: No statistically significant differences were detected between MON 87429 and the conventional control (α =0.05) in the combined-site, linear mixed model analysis. All plots at sites INSH, NESW, and NEYO were thinned to uniform density following early stand count.

† Indicates that p values could not be generated due to insufficient variability for formal statistical analysis.

[1] N = 32 for means. S.E. = standard error.

[2] Minimum and maximum mean values among 16 references, where each mean was combined over all the sites at which the reference was planted.

VII.C.2.2. Environmental Interactions Assessments

Plant responses to abiotic stressors, diseases, and arthropod pests were assessed at natural levels, i.e., no artificial infestation or imposed abiotic stress; therefore, these levels typically varied between observations at a site and among sites. These data were collected from each plot using a categorical scale (none, slight, moderate, and severe) of increasing severity of observed damage for each stressor. This scale was utilized to allow for the evaluation of the wide variety of potential abiotic stressors, diseases, and arthropod pests potentially occurring across the season and across sites. These data were summarized and not subjected to ANOVA. For a particular stressor, all comparisons of the range of responses for MON 87429 to the range of responses for the conventional control across all observation times and sites are reported. Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VII-1. The materials, methods, additional details concerning the qualitative environmental interactions assessments, and detailed results of the qualitative data comparisons are further discussed and presented in Appendix I.

In an assessment of plant responses to abiotic stressors, diseases, and arthropod pests, no differences were observed between MON 87429 and the conventional control for any of the 288 observations (including 96 abiotic stressor, 96 disease, and 96 arthropod pest observations) among all observations at eight sites (Table VII-5). These results support the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize.

Table VII-5. Summary of Qualitative Environmental Interactions Assessments for MON 87429 during 2017

Stressor	Number of observations across all sites	Number of observations with no differences between MON 87429 and the conventional control across all sites
Abiotic stressors	96	96
Diseases	96	96
Arthropod pests	96	96
Total	288	288

No differences were observed between MON 87429 and the conventional control during any of the 96 observations (8 sites, 4 timepoints, 3 stressors) for each stressor category. MON 87429 and the conventional control were considered different in susceptibility or tolerance if the range of injury symptoms across four replications did not overlap between MON 87429 and the conventional control.

VII.C.3. Pollen Characteristics

USDA-APHIS considers the potential for gene flow and introgression of the biotechnology-derived trait(s) into sexually compatible plants and wild relatives to be a factor in determining the potential for increased weedy or invasive characteristics of the receiving species. Pollen morphology and viability information are pertinent to this assessment and, therefore, were assessed for MON 87429. In addition, morphological characterization of pollen produced by MON 87429 and the conventional control is relevant to the plant pest risk assessment because it adds to the detailed description of the phenotype of MON 87429 compared to the conventional control.

The viability and morphology of pollen collected from MON 87429 compared to that of the conventional control were assessed. Pollen was collected from MON 87429, the conventional control, and four commercial references grown under similar agronomic conditions at a field site in Warren County, Illinois; a geographic area that represents environmentally relevant conditions for maize production for this product. The study was arranged in a randomized complete block design with four replications. Pollen was collected from three non-systematically selected plants per plot and stained for assessment. MON 87429 was compared to the conventional control for percentage viable pollen and pollen diameter. Descriptions of the evaluated pollen viability and morphology characteristics and the timing of the evaluations are listed in Table VII-1. The materials, methods, and general pollen morphology results are further discussed and presented in Appendix J.

No statistically significant differences (α=0.05) were detected between MON 87429 and the conventional control for percentage viable pollen or pollen diameter (Table VII-6). Furthermore, no visual differences in general pollen morphology were observed between MON 87429 and the conventional control (Appendix J, Figure J-1). These results support the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize.

Table VII-6. Viability and Diameter of Pollen Collected from MON 87429, the Conventional Control, and the Reference Materials

Pollen Characteristic (unit)	Mean (S.E.)[1]		Reference Range[2]
	MON 87429	Control	
Viability (%)	98.9 (0.37)	99.1 (0.32)	98.2 – 99.1
Diameter (µm)	85.1 (0.45)	85.1 (0.34)	83.4 – 86.7

Note: No significant differences were detected between the MON 87429 and the conventional control (α=0.05).
[1] MON 87429 and the conventional control values represent means with standard error (S.E.) in parentheses. N=4.
[2] Reference range is the minimum and maximum mean value observed among reference materials.

VII.D. Conclusions for Phenotypic, Agronomic, and Environmental Interactions Evaluation

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest potential as assessed by USDA-APHIS. Under the framework of familiarity, characteristics for which no statistically significant differences are detected support a conclusion of no increased plant pest potential of the biotechnology-derived crop compared to the conventional crop. Ultimately, a weight of evidence approach that considers all characteristics and data is used for the overall risk assessment of differences and their significance.

An extensive and robust set of phenotypic, agronomic, and environmental interactions data were used to assess whether the introduction of the dicamba, glufosinate, quizalofop, and 2,4-D-tolerance and RHS traits altered the plant pest potential of MON 87429 compared to the conventional control, considered within the context of the variation among the reference maize hybrids. These assessments included five general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development (including pollen characteristics); 4) lodging and seed retention on the plant; and 5) plant responses to abiotic stressors, diseases, and arthropod pests. Within these data categories, specific characteristics typically associated with weediness were also assessed to determine whether there was a potential increase in weediness of MON 87429 compared to conventional maize.

Results from these assessments comparing MON 87429 and the conventional control support the conclusion that MON 87429 does not possess: 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests; or 3) characteristics that would confer a plant pest risk compared to conventional maize. Therefore, based on the results of multiple assessments discussed above and presented in the appendices, the weight of evidence supports the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize and is unlikely to pose a plant pest risk.

VIII. U.S. AGRONOMIC PRACTICES

VIII.A. Introduction

As part of the plant pest assessment required by 7 CFR § 340.6(c)(4), impacts to agricultural and cultivation practices must be considered. This section provides a summary of current agronomic practices in the U.S. and North America for producing field maize and is included in this petition as a baseline to assess whether there is likely to be a significant change in agricultural practices due to the cultivation of MON 87429 maize and whether such changes are likely to exacerbate plant pests or diseases associated with maize. Discussions include maize production, plant growth and development, general management practices during the season, management of insects, diseases and weeds, crop rotation, and volunteer management. Information presented in Section VII.C demonstrated that MON 87429 maize is no more susceptible to diseases or pests than commercially cultivated maize. Additionally, data presented support that, with the exception of the introduced traits, MON 87429 maize is phenotypically similar to conventional maize and is not expected to pose a greater plant pest risk compared to conventional maize. Thus, except for greater diversity in herbicide chemistry tolerance that will provide greater flexibility in weed control options, there are no expected changes to the inputs needed for MON 87429 maize production, and no expected impacts to most of the agronomic practices employed for production of maize compared to the current practices.

Maize is planted in almost every U.S. state demonstrating its wide adaptation to soils and climate. However, the majority of maize is produced in the Midwest states because the fertile soils and climate are favorable for maize production. Proper seedbed preparation, good genetics, proper planting dates, plant population density, soil fertility, water availability and good integrated pest management practices are important to optimize the yield potential and economic returns of maize.

Annual and perennial weeds are considered to be the greatest pest problem in maize production (Aref and Pike, 1998). Weeds compete with maize for water, nutrients, and light resulting in substantial yield losses when left uncontrolled. Weed species in maize vary from region to region and state to state. Maintaining weed populations below economic thresholds in maize requires some form of weed management practice on all maize acreage. Weed management practices include mechanical practices (e.g., tillage), cultural practices (e.g., crop rotation, variety selection, optimizing planting date, plant population and row spacing), and chemical practices (e.g., herbicide application). Numerous herbicides are available for preplant, preemergence, and postemergence control of annual and perennial weeds in maize, and approximately 98% of the maize acreage in the U.S. receives an herbicide application (USDA-NASS, 2019b).

As shown in Sections VI and VII, with the exception of introduced traits, no biologically meaningful phenotypic, compositional, or environmental interaction differences between MON 87429 maize and conventional maize have been observed. Moreover, herbicide-tolerant maize is currently grown on approximately 90% of U.S. maize acres (USDA-ERS, 2018b). Dicamba, glufosinate, quizalofop, 2,4-D and glyphosate herbicides are

currently labeled for preplant and postemergence applications in maize. In support of Monsanto's third generation herbicide tolerant product MON 87419 maize, Monsanto requested that EPA allow the 0.5 lb a.e./ac postemergence application window for dicamba to be extended from V5 to V8 growth stage or 36-inch height of maize, whichever occurs first (U.S. EPA, 2019). The combined (pre- and post-emergence) maximum annual application rate of dicamba in MON 87419 maize would be 2.0 lbs. a.e. dicamba per acre per year, increased from the current maximum annual application rate of 0.75 lbs. a.e. in conventional maize. Pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of MON 87419. Glufosinate, quizalofop and 2,4-D herbicides are currently labeled for preplant applications on conventional and herbicide-tolerant maize hybrids and for in-crop postemergence applications on herbicide-tolerant (HT) hybrids. The intended preplant and postemergence uses of these herbicides with MON 87429 maize would not be any different than current labeled uses in HT maize and therefore does not require a label change. The MON 87429 event confers glyphosate tolerance in specific plant tissues (i.e., not in tassels) and will be used to facilitate the production of hybrid seed. MON 87429 is not intended to be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated events that confer full-plant glyphosate tolerance (e.g., NK603). The combination of herbicide-tolerance traits will allow the preplant and postemergence use of dicamba, glufosinate, quizalofop, 2,4-D and glyphosate herbicides in an integrated weed management program in maize with multiple sites-of-action to control a broad spectrum of grass and broadleaf weed species, including herbicide-resistant and tough to control weed species. Therefore, it is not anticipated that commercialization of MON 87429 maize in the U.S. would have a notable impact on current maize production practices, beyond the intended benefits of effective management of common, troublesome weeds, and/or herbicide-resistant weeds and additional options for growers to rotate and/or use combinations of herbicides with multiple sites-of-action for preplant and in-crop postemergence herbicide applications.

Hybridization is a fundamental concept used in maize breeding and production programs in the U.S. and most of the world. The fixation of alleles in pure lines (i.e., inbreds) causes a general reduction in maize vigor and productivity, but maize grown from the F1 hybrid seed produced through crossing two inbred lines has improved vigor (e.g., heterosis) compared to the open pollinated varieties. Modern maize breeding is based on selecting inbred lines and producing crosses that possess desirable traits. Techniques such as marker-assisted selection can also reduce the time and cost required to achieve breeding goals (Yousef and Juvik, 2001).

Tissue-specific expression of CP4 EPSPS protein in MON 87429, allowing for glyphosate induced non-viable pollen phenotype, is the second generation of Monsanto's Roundup® Hybridization System (RHS) for hybrid seed production. The first-generation RHS event, MON 87427 maize, was deregulated by USDA in 2013 (USDA-APHIS Petition #10-281-01p). Similar to the MON 87427 RHS trait, the second-generation RHS trait in MON 87429 allows inbred MON 87429 lines, treated with glyphosate at the appropriate timings (V8 to V13) to serve as female parents in the production of hybrid seed. Inbred MON 87429 lines receive two glyphosate applications at vegetative growth stages ranging from V8 to V13, resulting in the intended non-viable pollen phenotype due

to tissue-specific glyphosate tolerance. An additional benefit of including the RHS trait in MON 87429 maize at a single locus with dicamba, glufosinate, quizalofop and 2,4-D tolerance traits, is the reduction in the number of trait loci that would otherwise need to be managed and combined, via traditional breeding methods.

In hybrid maize production systems, inbred MON 87429 plants treated with glyphosate during tassel development will be pollinated by male pollen donor plants that contain a glyphosate tolerance trait (e.g., NK603). Male donor inbred plants are cultivated in proximity to female inbred MON 87429 plants and the male donor inbred plants are not impacted by the glyphosate applications due to full glyphosate tolerance in all tissues. This cross results in hybrid offspring (e.g., MON 87429 × NK603) with full-plant tolerance to glyphosate, both in vegetative and reproductive tissues, as well as tolerance to dicamba, glufosinate, quizalofop, and 2,4-D herbicides.

VIII.B. Overview of U.S. Maize Production

VIII.B.1. Maize Grain Production

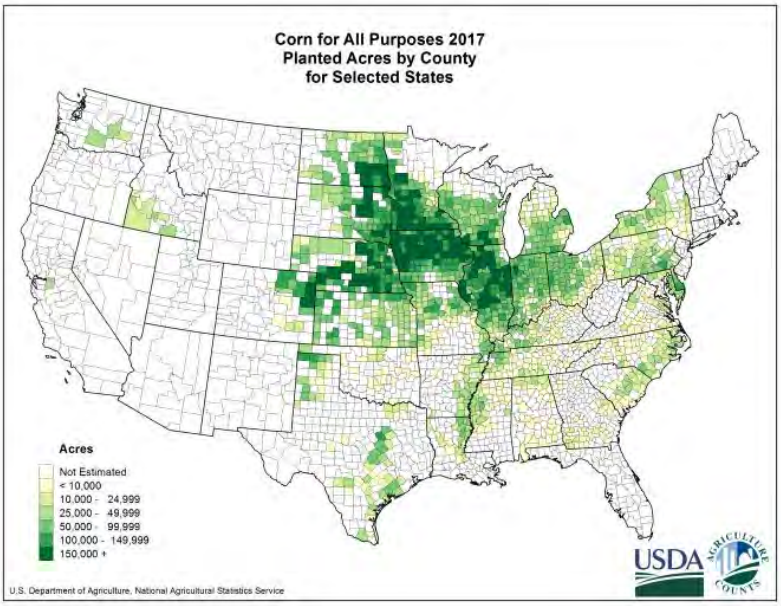
The U.S., China, Brazil, European Union and Argentina are the top five countries/regions producing maize globally (USDA-FAS, 2019). The U.S. is the largest producer of maize (*Zea mays*), producing approximately 35% of the world maize production in 2017/18 (USDA-FAS, 2019). China follows with approximately 24% of the maize production. Maize for all purposes was planted on approximately 89 million acres in 2018 in the U.S. (Table VIII-1), consistent with soybean acres and more than the 47.8 million wheat acres (USDA-NASS, 2019c). Much of that production occurs in the upper Midwest States (Figure VIII-1). The 2018 maize acreage was down 1.04 million acres from 2017 (Table VIII-1). Approximately 81.7 million acres were harvested for grain in 2018 and 6.1 million acres were harvested for silage in 2018 (USDA-NASS, 2019c). Total maize grain production was approximately 14.6 billion bushels in 2018 with an average yield of 178.6 bushels per acre (Table VIII-1). The value of maize grain production reached $48.46 billion in the U.S. in 2017 (Table VIII-1). The value of maize production in the U.S. has ranged from $46.73 to $76.94 billion in the past 10 years. The principal uses of maize are feed and residuals, ethanol fuel, exports, food, seed and industrial uses (Capehart et al., 2018).

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Table VIII-1. Field Maize Production in the U.S., 2007-2018[1]

Year	Acres Planted (×1000)	Acres Harvested (×1000)	Average Yield (bushels/acre)	Total Production (×1000 bushels)	Value (billions $)
2018	89,140	81,767	178.9	14,625,974	-[2]
2017	90,167	82,703	176.6	14,604,067	48.46
2016	94,004	86,748	174.6	15,148,038	51.30
2015	88,019	80,753	168.4	13,601,964	49.34
2014	90,597	83,136	171.0	14,215,532	52.95
2013	95,365	87,668	158.8	13,925,147	62.72
2012	97,155	87,375	123.4	10,780,296	74.33
2011	91,936	83,989	147.2	12,359,612	76.94
2010	88,192	81,446	152.8	12,446,865	64.64
2009	86,382	79,490	164.7	13,091,862	46.73
2008	85,982	78,570	153.9	12,091,648	49.31
2007	93,527	86,520	150.7	13,037,875	54.67

[1]Source: (USDA-NASS, 2018a)
[2]Data not available



Source:(USDA-NASS, 2018b)
Figure VIII-1. U.S. Maize Acreage by County in 2017

VIII.B.2. Maize Seed Production

Commercial hybrid maize seed production is a labor intensive process. Single cross hybrid maize seed production involves planting male and female parent inbreds in separate rows or blocks in an isolated field. The female parent inbred is prevented from shedding pollen to prevent self-pollination and ensure pollination only by the intended male parent inbred. The male parent inbred is usually destroyed by mechanical means

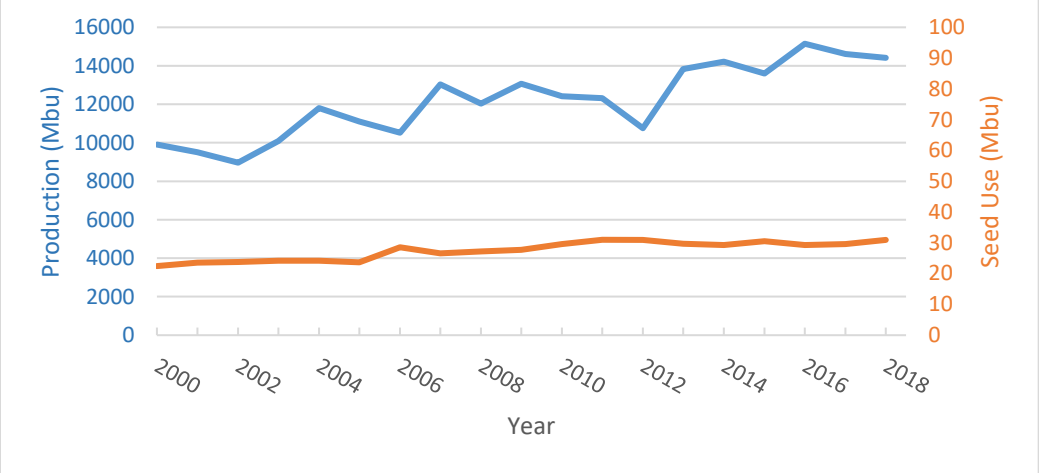
following pollination to prevent seed mixing during harvest. Ears that are produced from the cross-pollinated female parent inbred are harvested, processed, and the seed sold to farmers for planting as hybrid maize seed.

Maintaining an adequate supply of the parental inbred lines is vital to producing an adequate supply of hybrid maize seed. Often referred to as foundation seed, parental inbred lines are produced and maintained under strict isolation in the production field to preserve the identity and integrity of the genetics within each inbred. Quality control checks performed during the production of inbreds include visual inspections of the plants grown in isolation, and the use of molecular tools to verify the genetics of each inbred line (Gowda et al., 2017).

Maize seed production is not expected to be affected by the introduction of MON 87429. Over the last 18 years, the volume of hybrid maize seed planted in the U.S. has increased from 22.45 million bushels (MBu) planted in 2000 to 30.90 MBu planted in 2018 (USDA-ERS, 2018a). Grain yields have increased significantly over this same period from 9,915 MBu in the year 2000 to 14,420 MBu in 2018 (Figure VIII-2).

A number of factors must be considered in hybrid maize seed production, including: 1) forecasting the quantity of specific hybrid maize seed that will be needed at least a year in advance; 2) selection of a production area that mitigates risks and maximizes the yield of hybrid maize seed; and 3) agronomic practices. Key considerations and practices for producing hybrid maize seed are described in detail in MON 87427 USDA-APHIS Petition #10-281-01p (p. 123), the first generation RHS event and include: input considerations, control of weeds, disease and pests, plant density, plot isolation, parent delay to synchronize flowering, pollen control methods such as detasseling and cytoplasmic male sterility, harvesting and labeling requirements. These practices may differ from those used in commercial maize grain production.

The practices for producing hybrid maize seed using MON 87429 are generally similar to those using manual or mechanical detasseling methods or cytoplasmic male sterility (CMS), except for the use of glyphosate at early tassel development timings to confer the male sterile phenotype through tissue-specific glyphosate tolerance.



Source: (USDA-ERS, 2018a)

Figure VIII-2. Hybrid maize seed planted, and grain produced in the U.S. from 2000-2018.

VIII.C. Production Management Considerations

MON 87429 provides tolerance to the same herbicides (glufosinate, quizalofop and 2,4-D, and tissue-selective glyphosate) as several other events present in herbicide tolerant maize hybrids being grown in the U.S. (e.g., Genuity® SmartStax®, Genuity® VT Double PRO®, LibertyLink®, Enlist® maize, and first generation RHS MON 87427 maize), or for dicamba, events previously deregulated by USDA-APHIS (Petition #15-113-01p). With the widespread use of herbicide tolerant maize hybrids since 1997 (USDA-ERS, 2018b), it is anticipated that no major changes in production management practices will occur beyond the intended benefits of more effective and improved management of common, troublesome and/or herbicide-resistant weeds and the opportunity for growers to rotate and/or use combinations of herbicides with multiple sites-of-action for preemergence and in-crop postemergence herbicide applications. Cultivation practices for hybrid maize, containing the RHS trait in MON 87429, used in grain production will not differ from other commercially available, glyphosate tolerant maize.

The RHS trait in MON 87429 maize offers the same benefits to hybrid maize seed production as the RHS trait in MON 87427, described in detail in USDA-APHIS Petition #10-281-01p (p. 5). Briefly, these benefits include enabling maize hybrid seed producers to discontinue the practice of manually or mechanically detasseling female inbred plants in their production field, which must occur during a critical 3-4 day time period of maize tassel development that can be influenced by changes in weather (e.g., extreme heat). The ability to treat MON 87429 maize inbreds with glyphosate (between V8 to V13), in

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place of detasseling, provides flexibility to hybrid maize seed producers as well as reduces the cost of hybrid seed production by removing the reliance on costly, labor intensive manual/mechanical detasseling. For weed management during the production of hybrid seeds using MON 87429, seed producers will be provided best management practices for herbicide resistance management, including the use of herbicides with multiple sites-of-action. An additional benefit of including the RHS trait in MON 87429 maize at a single locus with herbicide tolerance traits is the reduction in the number of trait loci that would otherwise need to be managed and combined, via traditional breeding methods. Further, the amount of glyphosate recommended for use with MON 87429 maize for hybrid seed or grain production will not exceed the total amount of glyphosate already approved for in crop use with other commercially available, glyphosate tolerant maize events.

VIII.D. Management of Insect Pests

Monsanto summarized major issues associated with the management of insect pests in its petition for nonregulated status for corn rootworm-protected maize MON 87411 (USDA-APHIS Petition #13-290-01p). MON 87429 maize does not contain insect protection traits, therefore the information on this subject is incorporated here by reference (USDA-APHIS Petition #13-290-01p p. 166). In brief, insect pests continue to cause damage to maize and are commonly addressed by biotechnology-derived insect-tolerant traits, insecticide treatment of seeds, soil or foliar application of insecticides, or use of crop rotation or other integrated pest management practices. The EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), regulates the distribution, sale, use and testing of pesticidal substances (including those produced in plants), that are intended to control insect pests.

MON 87429 was developed to improve the management of weeds and has no unique insect pest control attributes. Environmental observations in field studies indicated that the presence of the dicamba, glufosinate, quizalofop, and 2,4-D-tolerance and RHS traits did not meaningfully alter the susceptibility of MON 87429 maize to arthropod-related damage (Section VII.C). Therefore, no changes in current insect pest management practices in maize are anticipated from the introduction of MON 87429.

VIII.E. Management of Diseases and Other Pests

Monsanto summarized major issues associated with the management of diseases and non-insect pests in its petition for nonregulated status for Corn Rootworm-Protected Maize MON 87411 (USDA-APHIS Petition #13-290-01p). MON 87429 does not contain disease protection traits, therefore the information on this subject is incorporated here by reference (USDA-APHIS Petition #13-290-01p p. 171). Briefly, management of diseases and pests of maize are important to protecting the yield of harvested grain. Disease and pest incidence vary from year to year and growers may choose to use pesticides or a variety of management practices to control problematic diseases or pests.

MON 87429 was developed to improve the management of weeds and has no unique attributes for control of diseases or other pests. Environmental observations in field studies indicated that the presence of the dicamba, glufosinate, quizalofop, and 2,4-D-

tolerance and RHS traits did not meaningfully alter the susceptibility of MON 87429 maize to diseases or other pests (Section VII.C). Therefore, no changes in current management practices for diseases or other pests in maize are anticipated from the introduction of MON 87429 maize.

VIII.F. Weed Management

VIII.F.1. Methods of Weed Control in Maize

Annual and perennial weeds are considered to be the greatest pest problem in maize production (Aref and Pike, 1998). Weed control in maize is essential for optimizing yield because weeds compete with maize for light, nutrients, and moisture and can lead to reductions in yield (Knake et al., 1990). The duration of competition from weeds is important to determine the potential loss of yield in maize and the critical time period can vary with the density and species of the weed and environmental factors (Hall et al., 1992). Early weed competition studies indicated that weeds must be removed by a certain time and maize heights to avoid yield losses in maize (Carey and Kells, 1995; Hall et al., 1992; Knake and Slife, 1965; Tapia et al., 1997). Weed control in the first several weeks after maize emergence is the most critical period to avoid yield losses in maize (Bosnic and Swanton, 1997; Carey and Kells, 1995; Hall et al., 1992; Knezevic and Datta, 2015). Some weeds can tolerate cold, wet conditions better than maize, and can gain an advantage prior to planting. A number of weeds compete with maize and reduce yield significantly with delay in weed control. Some of these weeds are difficult to control since they have similar life cycle and growth habit as those of maize plant (Jhala et al., 2014). Annual weed species such as giant foxtail (*Setaria faberi*), barnyardgrass (*Echinochloa crus-galli* (L.) *Beauv*.) and Palmer amaranth (*Amaranthus palmeri*) have been shown to reduce maize yields by up to 13% (Fausey et al., 1997; Knake and Slife, 1965), 35% (Bosnic and Swanton, 1997) and 91% (Massinga et al., 2003), respectively. A compilation of data from 2007-2013 on weeds impacting yield in North America showed that uncontrolled weeds in maize could reduce yield at an average of 50% annually (Soltani et al., 2016). In a study of mixed weed populations competing with maize, yields were reduced by up to 20% when the weeds reached a height of eight inches (Carey and Kells, 1995).

A case study on herbicide tolerant field maize within a larger survey involving Extension Service weed scientists solicited estimates of the percent of maize acreage infested with individual weed species by state or region, as well as the potential impact on maize yields if the species were left uncontrolled. In this survey, at least twelve annual broadleaf, nine annual grass, and seven perennial species were identified as troublesome weeds (Table VIII-2) (Gianessi et al., 2002). Estimates of yield loss in this case study ranged from a low of 15% due to wirestem muhly and sandburs to a high of 48% from bur cucumber.

Crop rotations and environment have a significant impact on the adaptation and occurrence of weeds in maize. A national weed survey conducted in 2015 has identified Palmer amaranth (*Amaranthus palmeri*), common lambsquarters (*Chenopodium album*), Canada thistle (*Cirsium arvense*), kochia (*Bassia scoparia*), common waterhemp (*Amaranthus rudis*), giant ragweed (*Ambrosia trifida*), morningglory spp (*Ipomoea* spp.), yellow nutsedge (*Cyperus esculentus*), common ragweed (*Ambrosia artimisiifolia*) and

downy brome (*Bromus tectorum*) as top 10 most troublesome weeds in the US (Van Wychen, 2015). Waterhemp, Palmer amaranth, morningglory, giant ragweed, horseweed and johnsongrass are common troublesome weeds in Midwest fields. The most frequently reported common weeds in the Southeast region are pigweeds (*Amaranthus* spp.), morningglory, crabgrass (*Digitaria* spp.), nutsedge (*Cyperus* spp.) and broadleaf signalgrass (*Brachiaria platyphylla*) (Webster et al., 2012). Morning glory, pigweeds, nutsedge, johnsongrass (*Sorghum halepense*), crabgrass and horseweed (*Conyza canadensis*) are the most frequently mentioned troublesome weeds in the Southeast region (Webster et al., 2012).

Until the early 1950s, tillage and cultivation practices were primarily used for weed control in maize, but they have been largely replaced by the use of herbicides. Herbicide use in maize became widespread by the end of the 1970s (Timmons, 2005). In 2018, herbicides were applied to 98% of the planted maize acreage (USDA-NASS, 2019b). Glyphosate is the most widely applied herbicide in maize being applied on [ ] of the planted acreage (Table VIII-3). Triketones and Isoxazole are the second most widely used group of herbicides at [ ] followed by chloroacetamide and isoxazoline herbicides at [ ] and Triazines (atrazine, metribuzin, simazine) at [ ] [ ]. CBI-Deleted CBI-Deleted CBI-Deleted, CBI-Deleted CBI-Deleted

In 2018, approximately 90% of the total maize acreage in the U.S. was planted with hybrids possessing biotechnology-derived herbicide-tolerance traits (USDA-ERS, 2018b). Maize hybrids possessing both herbicide-tolerance and insect-protected traits were planted on 80% of the maize acreage in 2018 (USDA-ERS, 2018b). The introduction of herbicide-tolerant crops such as Roundup Ready®, LibertyLink® and recently Enlist® maize, have offered growers an alternative and effective solution for the weeds control in maize by enabling in-crop postemergence use of glyphosate, glufosinate, quizalofop and 2,4-D herbicides. Although these herbicides (glyphosate, glufosinate, quizalofop and 2,4-D) provide control of numerous annual and perennial weed species, preemergence herbicides are a key component of weed control programs in maize. This is evident in the widespread use of triketones, chloroacetamide and triazine herbicides in maize (Table VIII-3, Table VIII-4 and Table VIII-5). Preemergence residual herbicides provide early season weed control to reduce early weed competition, improve control of certain hard to control broadleaf weed species (morningglory spp.), and help provide control of some glyphosate-resistant weeds.

Table VIII-4 and Table VIII-5 provide a summary of the crop tolerance ratings of herbicides applied in maize production and the efficacy of these herbicides on 26 common weed species. These tables list only the most commonly used herbicides in maize production. Seldom would one field or farm have all 26 weed species, but they generally have a mixture of grass and broadleaf weed species. These ratings can be used by growers to facilitate the selection of an herbicide program in maize, which offers the

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best overall control of the weed species present. Generally, a mixture or premixture of two or more herbicide active ingredients is needed to achieve broad spectrum control of both grasses and broadleaf weed species. However, glyphosate, glufosinate, tembotrione, and topramezone each provide control of numerous grass and broadleaf weed species.

Table VIII-2. Troublesome Weeds in Maize Production[1]

Weed Species ***Latin name***	**Area Infested State/Region[2]**	**Acreage Infested (%)**	**Potential Yield Loss (%)**
Annual Broadleaves			
Bur Cucumber (*Sicyos angulatus*)	PA/OH/TN/SE	5-10	48
Cocklebur (*Xanthiums strumarium*)	MW/NP/SE	20-60	33
Jimsonweed (*Datura stramonium*)	MW/CO	5-20	17
Kochia (*Bassia scoparia*)	NP/NW	10-70	33
Lambsquarters (*Chenopodium album*)	MW/SE/NE/CA	15-80	33
Morningglory (*Ipomoea* spp.)	MW/SE/SP	20-75	33
Nightshade (*Solanum* spp.)	MW/NP/CA	25-50	26
Pigweeds/Waterhemp (*Amaranthus* spp.)	US	30-90	36
Ragweed, Common (*Ambrosia artemisiifolia*)	MW/SE/NE	20-70	30
Ragweed, Giant (*Ambrosia trifida*)	MW/NP	10-45	28
Smartweeds (*Polygonum* spp.)	MW/SD/NE/SE	30-70	22
Velvetleaf (*Abutilon theophrasti*)	MW/NE/NP	25-70	28
Annual Grasses			
Barnyardgrass (*Echinochloa crus-galli*)	SP/NW/CA	80-90	23
Bermudagrass (*Cynodon dactylon*)	MD/SE/UT/CA	10-20	47
Crabgrass spp. (*Digitaria* spp.)	MW/SE/NE	20-80	29
Cupgrass, Woolly (*Eriochloa villosa*)	IA/WI	15-20	29
Foxtail spp. (*Setaria* spp.)	MW/NE/NP	50-90	31
Millet, Wild-Proso (*Panicum miliaceum*)	UT/WY/CO/ID	15-40	31
Panicum, Fall (*Panicum dichotomiflorum*)	MW/SE/NE/NP	15-80	30
Sandburs (*Cenchrus* spp.)	NP/UT/WY	5-30	15
Shattercane (*Sorghum bicolor*)	MW/SP	5-40	33
Perennials			
Bindweed, Field (*Convolvulus arvensis*)	ND/SW/CA	40-80	18
Dogbane, Hemp (*Apocynum cannabinum*)	IL/MO	2-20	21
Johnsongrass (*Sorghum halepense*)	MW/SE/SW/CA	20-60	45
Muhly, Wirestem (*Muhlenbergia frondosa*)	PA	2	15
Nutsedge, Yellow (*Cyperus esculentus*)	MW/SE/NE/NP/ CA	10-70	21
Quackgrass (*Elytrigia repens*)	MW/NE/UT	10-70	27
Thistle, Canada (*Cirsium arvense*)	NE/MW/NP/CO	5-25	26

[1]Source: (Gianessi et al., 2002).
[2]Regions: MW = Midwest, NE = Northeast, NP = Northern Plains, NW = Northwest, SE = Southeast, SW = Southwest, SP = Southern Plains.

Table VIII-3. Herbicide Applications in Maize in 2018 in the U.S.[1]

Herbicide	Chemical Family	Site-of-Action[2] (SOA)	Percent of Maize Acres Treated	Percent of Maize Acres Treated per SOA	
Glyphosate	Glycine	EPSPS inhibitor	[ ]	[ ]	CBI-Deleted, CBI-Deleted
Atrazine	Triazine	PSII inhibitor	[ ]	[ ]	CBI-Deleted
Metribuzin	Triazine		[ ]		CBI-Deleted, CBI-Deleted
Simazine	Triazine		[ ]		CBI-Deleted
Acetochlor	Chloroacetamide	Long-chain fatty acid inhibitor	[ ]	[ ]	CBI-Deleted
Alachlor	Chloroacetamide		[ ]		CBI-Deleted
Dimethenamid	Chloroacetamide		[ ]		CBI-Deleted, CBI-Deleted
Metolachlor	Chloroacetamide		[ ]		CBI-Deleted
Pyroxasulfone	Isoxazoline		[ ]		CBI-Deleted
Isoxaflutole	Isoxazole	HPPD inhibitor	[ ]	[ ]	CBI-Deleted
Mesotrione	Triketone		[ ]		CBI-Deleted
Tembotrione	Triketone		[ ]		CBI-Deleted, CBI-Deleted
Topramezone	Triketone		[ ]		CBI-Deleted
Bicyclopyrone	Triketone		[ ]		CBI-Deleted

Table VIII-3. Herbicide Applications in Maize in 2018 in the U.S.[1] (continued)

Herbicide	Chemical Family	Site-of-Action[2] (SOA)	Percent of Maize Acres Treated	Percent of Maize Acres Treated per SOA	
2,4-D	Phenoxy	Synthetic Auxin	[ ]	[ ]	CBI-Deleted
Clopyralid	Carboxylic acid		[ ]		CBI-Deleted
Dicamba	Benzoic acid		[ ]		CBI-Deleted, CBI-Deleted
Fluroxpyr	Caryridine Carboxylic acid		[ ]		CBI-Deleted
Flumetsulam	Imidazolinone	ALS inhibitor	[ ]	[ ]	CBI-Deleted
Halosulfuron	Sulfonylurea		[ ]		CBI-Deleted
Nicosulfuron	Sulfonylurea		[ ]		CBI-Deleted
Primisulfuron	Sulfonylurea		[ ]		CBI-Deleted
Prosulfuron	Sulfonylurea		[ ]		CBI-Deleted, CBI-Deleted
Rimsulfuron	Sulfonylurea		[ ]		CBI-Deleted
Thifensulfuron	Sulfonylurea		[ ]		CBI-Deleted
Thiencarbazone	Triazolones		[ ]		CBI-Deleted
Tribenuron	Sulfonylurea		[ ]		CBI-Deleted

Table VIII-3. Herbicide Applications in Maize in 2018 in the U.S.[1] (continued)

Herbicide	Chemical Family	Site-of-Action[2] (SOA)	Percent of Maize Acres Treated	Percent of Maize Acres Treated per SOA
Diflufenzopyr	Semicarbazone	Auxin transport	[ ]	[ ]
Fluthiacet	Thiadiazole	PPO inhibitor	[ ]	[ ]
Carfentrazone	Aryl triazone		[ ]	
Saflufenacil	Pyrimidinedione		[ ]	
Flumioxazin	N-phenylphthalimide		[ ]	
Paraquat	Bipyridylium	Photosystem-I-electron diverter	[ ]	[ ]
Glufosinate	Phosphinic acid	Glutamine Synthase Inhibitor	[ ]	[ ]
Pendimethalin	Dinitroanaline	Microtubule inhibitor	[ ]	[ ]

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[1][ ]
[2](WSSA, 2018)

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Table VIII-4. Crop Tolerance and Grass Weed Responses to Herbicides Applied in Maize Production

		Common Grass Weeds[1,2]											
Herbicide/Application	CT[3]	BY	BS	CG	FP	FTg	FTy	GG	SC	JGr	JGs	IR	NSy
					Preplant or Preemergence								
Acetochlor	1	9	NA	9	8	9	9	NA	-	NA	NA	NA	8+
Acetochlor/atrazine	1	9	8	9	8	9	9	9	-	0	7	8	8+
Acetochlor/flumetsulam/ clopyralid	2	8	NA	8	8	8	8	NA	-	NA	NA	NA	7
Atrazine	0	8	5	-	-	7	7	6	-	0	4	NA	7
Dimethenamid	1	8	NA	8+	8	8+	8+	NA	-	NA	NA	NA	8
Flumetsulam	2	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Flumetsulam/clopyralid	2	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Flumioxazin	1	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Flumioxazin/pyroxasulfone	1	8	8	8	8	8	8	8	-	3	9	9	-
Isoxaflutole	1	8	NA	7	8	8	6	NA	6	NA	NA	NA	-
Mesotrione	1	-	NA	6	-	-	-	NA	-	NA	NA	NA	-
Metolachlor	1	8	NA	9	8+	9	9	NA	-	NA	NA	NA	8+
Metolachlor/atrazine	1	9	8	9	8	9	9	9	-	0	7	8	8
Metolachlor/mesotrione	1	8	NA	9	8+	9	9	NA	-	NA	NA	NA	8+
Metolachlor/mesotrione/atr	1	9	8	9	8+	9	9	9	-	2	8	7	8
Pyroxasulfone	1	8	8	8	8	9	8	9	-	4	6	9	-
Pyroxasulfone/fluthiacet	1	8	8	8	8	9	8	9	-	4	7	9	-
Pyroxasulfone/fluthiacet/atr	1	8	8	8	8	9	8	9	-	4	7	9	6
Rimsulfuron/thifensulfuron	1	7	NA	6	6	7	7	NA	-	NA	NA	NA	-
Rimsulfuron/isoxaflutole	2	8	NA	7	8	8	7	NA	6	NA	NA	NA	-
Rimsulfuron/mesotrione	1	7	NA	6	6	7	7	NA	-	NA	NA	NA	-
Saflufenacil	1	-	1	-	-	-	-	1	-	NA	1	1	-
Saflufenacil/Dimethenamid	1	8	6	8	8	8	8	8	-	NA	NA	NA	-
Simazine	0	8	5	7	7	8	8	7	-	0	4	NA	-
					Preemergence								
Thiencarbazone/isoxaflutole	1	8	8	8+	8+	8+	8+	9	7	NA	NA	NA	7
Pendimethalin	2	8	6	8	8	8	8	8	6	4	7	5	-

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Table VIII-4. Crop Tolerance and Grass Weed Responses to Herbicides Applied in Maize Production (continued)

Herbicide/Application	CT[3]	Common Grass Weeds[1,2]											
		BY	BS	CG	FP	FTg	FTy	GG	SC	JGr	JGs	IR	NSy
		Postemergence											
2,4-D	2	-	0	-	-	-	-	1	-	0	0	0	-
2,4-D/atrazine	2	-	NA	-	-	6	6	NA	-	NA	NA	NA	-
Atrazine	1	7	7	-	-	8	8	6	-	0	3	NA	7
Bentazon	0	-	0	-	-	-	-	1	-	0	0	0	8
Bromoxynil	1	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Carfentrazone	2	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Clopyralid	0	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Dicamba	2	-	1	-	-	-	-	1	-	0	0	0	-
Dicamba/diflufenzopyr	1	6	4	6	6	6	6	3	-	0	5	0	-
Fluroxypyr	1	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Flumiclorac	2	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Fluroxypyr/clopyralid	1	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Fluthiacet	2	-	NA	-	-	-	-	NA	-	NA	NA	NA	-
Glufosinate	0*	7	8	8	8	8+	7	5	8	7	8	6	-
Glyphosate	0*	8	9	8	8	9	9	9	9	7	9	6	7
Halosulfuron	1	-	2	-	-	-	-	2	-	1	2	NA	9
Mesotrione	1	-	7	7	-	-	-	NA	-	0	0	NA	-
Nicosulfuron	1	8+	8	4	8+	9	9	NA	9	8	9	6	6
Nicosulfuron/rimsulfuron	1/2	8	NA	-	8	9	9	NA	9	NA	NA	NA	-
Primisulfuron	2	-	NA	-	8	7	7	NA	9	NA	NA	NA	6
Primisulfuron/dicamba	2	-	NA	-	7	6	6	NA	9	NA	NA	NA	-
Prosulfuron/primisulfuron	2	-	NA	-	7	6	6	NA	9	NA	NA	NA	-
Rimsulfuron/ thifensulfuron	1	7	NA	-	7	7	7	NA	7	NA	NA	NA	-
Rimsulfuron/mesotrione	1	7	7	-	7	7	7	8	7	7	9	4	-
Tembotrione	0	8	8	6	-	7	9	7	8	5	6	NA	-
Thiencarbazone/tembotrione	1	8	8	8	8	8+	9	NA	8	5	7	NA	-
Topramezone	0	7	6	7+	6	7+	7	7	6	4	7	0	-

[1]All weed control ratings except for BS, GG, JGr, JGs and IR are from the 2014 Weed Control Guide for Ohio and Indiana, Ohio State University and Purdue University (Loux et al., 2014). Ratings for BS, GG, JGr, JGs and IR are from the 2015 Weed Control Guidelines for Mississippi, Mississippi State University (Mississippi State University, 2015). Weed control rating for weeds, except BS, GG, JGr, JGs and IR, are: 9 = 90% to 100%, 8 = 80% to 90%, 7 = 70% to 80%, 6 = 60% to 70%, - = less than 60% control, not recommended. Weed control ratings for BS, GG, JGr, JGs and IR are: 9-10 = excellent, 7-8 = good, 4-6 = fair, 0-3 = none to slight, NA= data not available in (Mississippi State University, 2015). Ratings assume the herbicides are applied in the manner suggested in the guidelines and according to the label under optimum growing conditions.

[2]Weed species: BY = barnyardgrass, BS = broadleaf signalgrass, CG = crabgrass, FP = fall panicum, FTg = giant foxtail, FTy= yellow foxtail, GG = goosegrass, SC = shattercane, JGr = rhizome johnsongrass, JGs = seedling johnsongrass, IR = Italian ryegrass, and NSy = yellow nutsedge.

[3]Crop tolerance (CT) rating: 0 = excellent, 1 = good, 2 = fair, 3 = poor. Source: Loux et al., 2014.

*Rating based on glufosinate to Liberty Link® maize and glyphosate applied to Roundup Ready® Corn 2 maize.

Table VIII-5. Broadleaf Weed Responses to Herbicides Applied in Maize Production

	Common Broadleaf Weeds[1,2]													
Herbicide/Application	BN	CB	CR	GR	LQ	MG	HS	PA	PW	PS	SP	SW	VL	WH
					Preplant or Preemergence									
Acetochlor	8+	-	7	-	7+	-	NA	NA	8+	NA	NA	-	-	8
Acetochlor/atrazine	9	8	9	8	9	8	6	9	9	8	6	9	8	9
Acetochlor/flumetsulam/clopyralid	8+	8	8+	7+	9	6	NA	NA	9	NA	NA	8+	8+	8
Atrazine	9	8	9	8	9	8	7	9	9	8	8	9	8	9
Dimethenamid	8+	-	-	-	6	-	NA	NA	8	NA	NA	-	-	8
Flumetsulam	8	7	7	-	9	-	NA	NA	9	NA	NA	8	8+	-
Flumetsulam/clopyralid	8+	8	8+	7+	9	6	NA	NA	9	NA	NA	8+	9	-
Flumioxazin	9	-	7	-	9	7	NA	NA	9	NA	NA	7	7	7
Flumioxazin/pyroxasulfone	9	-	8	-	9	7	8	9	9	8	7	7	7	8
Isoxaflutole	9	-	9	6	9	-	NA	NA	9	NA	NA	8	9	8
Mesotrione	9	7	7	6	9	6	NA	NA	9	NA	NA	9	9	9
Metolachlor	8	-	-	-	6	-	NA	NA	8	NA	NA	-	-	8
Metolachlor/atrazine	9	8	9	8	9	8	6	9	9	8	6	9	8	9
Metolachlor/mesotrione	9	7	7	6	9	6	NA	NA	9	NA	NA	9	9	9
Metolachlor/mestrione/atr	9	8	9	8	9	8	8	9	9	9	8	9	9	9
Pyroxasulfone	8	-	7	-	8	-	3	9	8	7	NA	-	7	8
Pyroxasulfone/fluthiacet	8		7	-	8	-	3	9	8	7	NA	-	7	8
Pyroxasulfone/fluthiacet/atr	9	7	9	6	9	7	7	9	9	8	8	9	8	9
Rimsulfuron/ thifensulfuron	-	-	7	-	7	7	NA	NA	7	NA	NA	7	6	-
Rimsulfuron/isoxaflutole	8	-	8	6	9	7	NA	NA	9	NA	NA	8	9	8
Rimsulfuron/mesotrione	9	7	8	6	9	6	NA	NA	9	NA	NA	9	9	9
Saflufenacil	8	8	8	8	9	8	6	9	9	7	5	8	8	8
Saflufenacil/Dimethenamid	9	8	9	8	9	8	6	9	9	7	5	9	8	9
Simazine	9	7	9	7	9	7	NA	9	9	9	8	8+	7	-
Thiencarbazone/isoxaflutole	9	8	9	8	9	7	9	NA	9	NA	NA	9	9	9
					Preemergence									
Pendimethalin	-	-	-	-	8	-	0	7	9	0	0	-	-	8

Table VIII-5. Broadleaf Weed Responses to Herbicides Applied in Maize Production (continued)

Herbicide/Application	Common Broadleaf Weeds[1,2]													
	BN	CB	CR	GR	LQ	MG	HS	PA	PW	PS	SP	SW	VL	WH
					Postemergence									
2,4-D	7	9	9	9	9	9	8	8	9	8	8	6	8	8
2,4-D/atrazine	9	9	9	9	9	9	NA	NA	9	NA	NA	9	8+	9
Atrazine	9	9	9	8	9	9	7	9	9	9	8	9	8	9
Bentazon	-	9	7	6	6	-	4	3	-	8	1	9	8+	-
Bromoxynil	9	9	9	8	9	8	NA	NA	7	NA	NA	8	8	6
Carfentrazone	8	-	6	-	7	8	NA	NA	8+	NA	NA	-	9	7
Clopyralid	8	9	9	9	-	-	NA	NA	-	NA	NA	-	-	-
Dicamba	8	9	9	9	8	9	9	8	8	8	8	8	7+	8
Dicamba/diflufenzopyr	8	9	9	9	9	9	9	9	9	9	9	8+	8	8
Fluroxypyr	7	8	9	-	-	9	NA	NA	-	NA	NA	7	8	-
Flumiclorac	-	7	7	-	7	-	NA	NA	9	NA	NA	-	9	7
Fluroxypyr/clopyralid	7	9	9	9	-	9	NA	NA	-	NA	NA	7	8	-
Fluthiacet	-	-	-	-	7	7	NA	NA	8	NA	NA	-	9	7
Glufosinate	9	9	9	9	8	8	9	8	8	9	9	9	8	8
Glyphosate	8	9	8+	8+	8+	6	6	9	9	7	8	8	8	8
Halosulfuron	-	9	8	8	-	6	8	6	9	7	5	7	8	-
Mesotrione	9	7+	7	8	9	7	NA	9	8	9	5	9	9	9
Nicosulfuron	-	-	-	-	-	8	7	6	9	4	5	8	-	7
Nicosulfuron/rimsulfuron	-	6	-	-	-	6	NA	NA	9	NA	NA	7	-	-
Primisulfuron	8	9	9	9	-	6	NA	NA	9	NA	NA	8	8	-
Primisulfuron/dicamba	9	9	9	9	9	8	NA	NA	9	NA	NA	9	8+	8
Prosulfuron/primisulfuron	8	9	9	9	6	7	NA	NA	9	NA	NA	8+	8+	-

Table VIII-5. Broadleaf Weed Responses to Herbicides Applied in Maize Production (continued)

	Common Broadleaf Weeds[1,2]													
Herbicide/Application	BN	CB	CR	GR	LQ	MG	HS	PA	PW	PS	SP	SW	VL	WH
Rimsulfuron/ thifensulfuron	-	6	6	-	7	-	NA	NA	8	NA	NA	6	7	-
Rimsulfuron/mesotrione	9	8	8	8	9	7	NA	8	9	9	7	9	9	9
Tembotrione	9	8	8	8	9	7	NA	9	9	7	7	8	9	9
Thiencarbazone/tembotrione	9	8	8	8	9	7	NA	9	9	7	7	8	9	9
Topramezone	9	8	7	7	9	7	7	8	9	9	6	8	9	9

[1]All weed control ratings except for HS, PA, PS, and SP are from the 2014 Weed Control Guide for Ohio and Indiana, Ohio State University and Purdue University (Loux et al., 2014). Ratings for HS, PA, PS, and SP are from the 2015 Weed Control Guidelines for Mississippi, Mississippi State University (Mississippi State University, 2015). Weed control ratings for weeds, except HS, PA, PS, and SP, are: 9 = 90% to 100%, 8 = 80% to 90%, 7 = 70% to 80%, 6 = 60% to 70%, - = less than 60% control, not recommended. Weed control ratings for HS, PA, PS, and SP are: 9-10 = excellent, 7-8 = good, 4-6 = fair, 0-3 = none to slight, NA= data not available in (Mississippi State University, 2015). Ratings assume the herbicides are applied in the manner suggested in the guidelines and according to the label under optimum growing conditions.

[2]Weed species: BN = black nightshade, CB = cocklebur, CR = common ragweed, GR = giant ragweed, LQ = lambsquarters, MG = morningglory spp., HS = hemp sesbania, PA = palmer and spiny amaranth, PW = pigweed, PS= prickly sida, SP = sicklepod, SW = smartweed, VL = velvetleaf, and WH = waterhemp.

*Rating based on glufosinate to Liberty Link® maize and glyphosate applied to Roundup Ready® Corn 2 maize.

VIII.F.2. Herbicide Resistant Weeds in Maize

Table VIII-6 provides a summary of the common weeds in maize that have biotypes reported resistant to the various herbicide sites-of-action in the U.S. To date there are only two weed species with biotypes confirmed to be resistant to dicamba in the U.S. after over 40 years of use – kochia (*Bassia scoparia*) and prickly lettuce (*Lactuca serriola*) (Heap, 2019). Additionally, a population of lambsquarters (*Chenopodium album*) has been confirmed as resistant to dicamba in New Zealand, common hempnettle (*Galeopsis tetrahit*), kochia and wild mustard (*Sinapis arvensis*) in Canada, smooth pigweed (*Amaranthus hybridus*) in Argentina, wild mustard in Turkey and bachelor's button/cornflower *(Centaurea cyanus)* in Poland have been confirmed as resistant, for a total of 7 species worldwide with confirmed resistance to dicamba (Heap, 2019). Currently in the U.S., six grass species (of which three are common weeds in maize; Table VIII-6) and 11 broadleaf species (of which eight are common weeds in maize; Table VIII-6) have been confirmed to have resistance to glyphosate (Heap, 2019). Dicamba provides good to excellent control of all eight of the common broadleaf species resistant to glyphosate. These broadleaf weed species, except kochia biotypes in Kansas state, have not been reported to have populations that are resistant to both glyphosate and dicamba in the U.S. (Heap, 2019). There are a total of 3 weed species worldwide with biotypes that have resistance to glufosinate. The first species resistant to glufosinate in the U.S. was recently confirmed in a glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) population (Avila-Garcia and Mallory-Smith, 2011). Additionally, Italian ryegrass, perennial ryegrass (*L. perenne*) populations in New Zealand (Ghanizadeh et al., 2015), goosegrass (*Eleusine indica*) from Malaysia (Seng et al., 2010) and rigid ryegrass (*L. rigidum*) in Greece (Travlos et al., 2018) have been confirmed resistant to glufosinate. For quizalofop herbicide, johnsongrass (*Sorghum halepense*), Italian ryegrass, giant foxtail (*Setaria faberi*), cheatgrass (*Bromus tectorum*) and common wild oat (*Avena fatua*) have been reported to be resistant in the US (Heap, 2019); and for 2,4-D herbicide, a total of 5 broadleaf weeds (waterhemp, Palmer amaranth, prickly lettuce, wild carrot (*Daucus carota*) and buckhorn plantain (*Plantago lanceolata*) have been confirmed to be resistant in the US (Heap, 2019). A discussion regarding the usefulness of MON 87429 in management of herbicide resistant weeds can be found in Section VIII.F.4. The potential for development of weeds resistant to dicamba, glufosinate, quizalofop, 2,4-D and glyphosate resistance can be found in Appendix K.

Table VIII-6. Common Weeds in Maize and Their Resistance to Herbicide Sites-of-action in the U.S.[1]

	Site-of-action											
Weed Species[2]	**ACCase Inhibitors**	**ALS Inhibitors**	**Long Chain Fatty Acid Inhibitors**	**Microtubule Inhibitors**	**EPSPS Inhibitors**	**Photosystem II Inhibitors –C1**	**HPPD Inhibitors**	**PSII Inhibitors – C2**	**Synthetic Auxins**	**PS1 Electron Diverter**	**PPO Inhibitors**	**Glutamine Synthase Inhibitors**
Grasses												
Barnyardgrass	X[3]	X				X		X	X			
Crabgrass spp. (large, smooth)	X								X			
Foxtail spp. (giant, green, yellow)	X	X		X		X						
Italian ryegrass	X	X	X		X					X		X
Goosegrass				X	X	X				X	X	
Johnsongrass	X	X		X	X							
Shattercane		X										
Yellow nutsedge		X										
Broadleaves												
Black nightshade (Eastern)		X				X						
Common chickweed		X										
Common cocklebur		X										
Common purslane						X		X				
Common ragweed		X			X	X					X	
Giant ragweed		X			X							
Horseweed (marestail)		X			X	X		X		X		
Jimsonweed						X						
Kochia		X			X	X		X	X			
Lambsquarters		X				X						
Palmer amaranth		X	X	X	X	X	X		X		X	
Prickly sida		X										
Pigweed spp. (waterhemp, redroot, smooth, Powell,)		X	X		X	X	X	X	X		X	
Russian thistle		X			X							
Smartweed spp. (Pennsylvania, ladysthumb)						X						
Sunflower		X			X							
Velvetleaf						X						

[1] (Heap, 2019). [2]Weed species and herbicide sites-of-action listed are only those common in maize.
[3]Indicates confirmed resistance in the U.S.

VIII.F.3. Impact of MON 87429 Maize Introduction on Weed Management Practices

Monsanto has developed a new herbicide-tolerant MON 87429 maize, that can provide growers additional options for an effective and sustainable weed management system. MON 87429 maize offers dicamba, glufosinate, quizalofop and 2,4-D-tolerance, and enables RHS hybrid seed production. For commercial use, MON 87429 maize will be combined with deregulated glyphosate tolerance traits, such NK603, via traditional breeding techniques to provide full-plant tolerance to glyphosate. The combination of herbicide-tolerance traits will allow the preplant and postemergence use of dicamba, glufosinate, quizalofop, 2,4-D, and glyphosate herbicides in an integrated weed management program to control a broad spectrum of grass and broadleaf weed species (Ganie and Jhala, 2017; Johnson et al., 2010). Dicamba, glufosinate and 2,4-D will provide additional options to control hard to control broadleaf weeds in addition to glyphosate (e.g., hemp sesbania, morningglory species, and prickly sida) and these herbicides (dicamba, glufosinate and 2,4-D) also offer an effective control option for glyphosate-resistant broadleaf weed species, namely marestail, common ragweed, giant ragweed, Palmer amaranth and waterhemp when used according to the label (Ganie and Jhala, 2017; Johnson et al., 2010). These herbicides will also offer an effective control option for broadleaf species resistant to acetolactate synthetase (ALS) and protoporphyrinogen oxidase (PPO) chemistries. Similarly, quizalofop and glufosinate herbicides, in addition to glyphosate provide options to control grass weeds effectively, when used according to the label. With the introduction of MON 87429 maize, growers will have the ability to continue to use established maize production practices including crop rotation, tillage systems, labeled herbicides, and row spacing, thereby using the same planting and harvesting machinery currently being utilized. As MON 87429 maize will be stacked with deregulated glyphosate tolerance traits, such as NK603, to provide full plant tolerance to glyphosate, growers will also continue to have the flexibility and simplicity in weed control provided by glyphosate that will allow growers to continue to reap the environmental benefits associated with the use of conservation-tillage that is facilitated by the use of glyphosate for postemergence weed control (Monsanto, 2017).

Dicamba is currently authorized by U.S. EPA for preplant and/or early postemergence application in conventional maize. In support of Monsanto's third generation herbicide tolerant product MON 87419 maize, Monsanto requested that EPA allow the 0.5 lb a.e./ac postemergence application window for dicamba to be extended from V5 to V8 growth stage or 36-inch height of maize, whichever occurs first (U.S. EPA, 2019). The combined (pre- and post-emergence) maximum annual application rate of dicamba in MON 87419 maize would be 2.0 lbs. a.e. dicamba per acre per year, increased from the current maximum annual application rate of 0.75 lbs. a.e. in conventional maize. Pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of MON 87419. Growers will be able to apply dicamba alone or tank mixed with glyphosate or in sequence with glyphosate or glufosinate on MON 87429 maize. Pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of MON 87419. Glufosinate is currently authorized by U.S. EPA and labeled for preplant applications prior to planting or prior to emergence on conventional and herbicide-tolerant maize hybrids and for in-crop postemergence applications on glufosinate-tolerant hybrids only (Bayer CropScience, 2016). Glufosinate use in MON 87429 maize will not change from current labeled uses of glufosinate. Growers will be able to apply glufosinate alone and in sequence with dicamba, 2,4-D or quizalofop for preplant or postemergence in-crop applications on MON 87429 maize.

Over-the-top postemergence application rates and timings for glufosinate alone would be the same as currently labeled for glufosinate use in glufosinate-tolerant hybrids (i.e., from emergence up to the V7 growth stage at up to 0.402 lbs. a.i./acre, seasonal maximum of 0.80 lbs. a.i. per acre) (Bayer CropScience, 2016).

Quizalofop herbicide can cause severe crop injury in conventional maize. U.S. EPA has authorized the use of quizalofop on herbicide-tolerant maize for over-the-top application from V2 through V6 stages of maize development for managing grass weeds (DuPont, 2018). Quizalofop use in MON 87429 maize will not change from current labeled uses of quizalofop on herbicide-tolerant maize.

2,4-D has been used for many decades as preplant and postemergence herbicide for control of broadleaf weeds in maize fields and many other weed control applications including other crops, pastures and rangeland. 2,4-D with relative selectivity for broadleaf plants, can cause injury to conventional maize depending on the growth stage and method of application. U.S. EPA has authorized the use of 2,4-D with choline salt for over-the-top application on herbicide-tolerant maize (Dow, 2017) up to the V8 stage of maize development while keeping the total maximum seasonal rate per acre same as its use in conventional maize. 2,4-D use in MON 87429 maize is not anticipated to change from current labeled uses of 2,4-D on herbicide-tolerant maize.

The MON 87429 event confers glyphosate tolerance in specific plant tissues (i.e., not in tassels) and will be used to facilitate the production of hybrid seed. MON 87429 is not intended to be offered for commercial use as a stand-alone product, but will be combined, through traditional breeding methods, with other deregulated events that confer full-plant glyphosate tolerance (e.g., NK603). The general weed management recommendations for all the regions in the U.S. are shown in (Table VIII-7). A preemergence residual herbicide is recommended regardless of tillage system to 1) reduce early weed competition, and 2) to increase the likelihood that multiple effective herbicide sites-of-action are used in maize and to provide protections against additional resistance development to existing maize herbicides. The variability in herbicide recommendations will be dictated by variations in weed spectrum, tillage systems, and environment across these regions and is consistent with Monsanto and academics recommendations for a comprehensive weed resistance management program. These recommendations shown in Table VIII-7 are a high-end estimate of anticipated herbicide use associated with MON 87429 maize combined with glyphosate-tolerant maize.

Table VIII-7. Anticipated Weed Management Recommendations for MON 87429 Maize Combined with Glyphosate-Tolerant Maize Systems[1]

Application Timing	Conventional Tillage		Conservation Tillage (No-till or reduced till)	
	Hard to Control Weeds[2]	**GR[3] Weeds and Hard to Control Weeds[5]**	**Hard to Control Weeds[2]**	**GR[3] Weeds and Hard to Control Weeds[5]**
Preemergence (burndown, at planting)	Residual	Residual + Dicamba or 2,4-D	Glyphosate + Residual	Glyphosate + Residual + Dicamba or 2,4-D
Postemergence[4]	Glyphosate or glufosinate +[4] Dicamba or 2,4-D	Glyphosate or Glufosinate +[4] Dicamba or 2,4-D and/or Quizalofop	Glyphosate or Glufosinate + residual +[4] Dicamba or 2,4-D	Glyphosate or Glufosinate +[4] Dicamba or 2,4-D and/or Quizalofop

[1] The anticipated use patterns represent a high-end estimate for potential dicamba, 2,4-D, glufosinate and/or quizalofop use associated with MON 87429 combined with glyphosate-tolerant maize. Actual weed control practices by growers will vary depending on the specific weed spectrum, agronomic situation of the individual maize field and herbicide label recommendations.
[2] Hard to control weeds namely, morningglory species, hemp sesbania, prickly sida¸ and wild buckwheat.
[3] GR = glyphosate resistant.
[4] + = premix, tank mix or sequential application of herbicides.
[5] Dicamba and 2,4-D have broadleaf selectivity and quizalofop has grass selectivity. Follow label recommendations and best management practices for herbicide rotations. Recommendations for all fields will assume GR weeds are present.

As a result of introducing MON 87429, there are no major anticipated changes in the production of hybrid maize seed with the exception of the intended non-viable pollen phenotype resulting from glyphosate applications during reproductive development. Glyphosate can also be used for weed control in MON 87429 inbred seed increases or hybrid seed production (may result in non viable pollen phenotype). Other agricultural management practices for the production of hybrid maize seed and for the cultivation of commercial maize would also be no different for MON 87429 than for conventional maize hybrids.

Upon stacking of MON 87429 maize with deregulated glyphosate tolerance traits, such as NK603, growers will have the ability to continue use of established maize production practices including tillage systems; the same planting and harvesting machinery; established practices for management of insects, diseases, and other pests; and many of the current herbicides used for weed control, including glyphosate with its established environmental benefits.

VIII.F.4. MON 87429 Maize as a Component in Weed Resistance Management

Although herbicide resistance may eventually occur in a weed species when an herbicide is widely used, resistance can be delayed, contained, and managed through good management practices, research and education. Crops engineered with tolerance to established herbicides combined with tolerance to glyphosate may provide more diverse weed management options (Pallett, 2018). The combination of dicamba, glufosinate, quizalofop and 2,4-D herbicide-tolerance traits with glyphosate-tolerant maize, will facilitate the utilization of multiple herbicide sites-of-action in a grower's weed control system, and thereby reduce the potential for further resistance development to glyphosate, dicamba, glufosinate, quizalofop and 2,4-D herbicides as well as other important maize herbicides. Research conducted by Monsanto showed the value of multiple preplant herbicides (including soil active herbicides) followed by an early postemergence application of a mixture of herbicides with multiple sites-of-action, including a soil active herbicide, against broadleaf weeds that included species in the genus *Amaranthus* (Bayer CropScience, 2019b). Such a program would optimize the likelihood of using two or more effective sites-of-action against the targeted weeds. In areas with glyphosate-resistant and hard to control broadleaf weed populations, dicamba or 2,4-D may be applied pre-planting as a tank mix with preemergence herbicides and/or applied post-emergence in addition to glufosinate in MON 87429 maize. For control of glyphosate-resistant grass weed species, quizalofop provides a post-emergence option and may also be applied in sequence with glufosinate. The herbicide tolerance traits in MON 87249 maize also increase the potential to target late-emerging weed cohorts and weeds resistant to multiple herbicides with multiple herbicide sites-of-action.

Stewardship of dicamba, glufosinate, quizalofop and 2,4-D herbicides to preserve their usefulness to growers is an important aspect of Monsanto's stewardship commitment, as is discussed in Appendix K. Specifically, Monsanto has implemented and will continue to develop and proactively provide recommended weed resistance management practices[9], and will utilize multiple methods to distribute technical and stewardship information to growers, academics, and grower advisors through a variety of communication tools. Monsanto's Technology Use Guide (TUG) (Monsanto, 2019) will set forth the requirements and best practices for the cultivation of MON 87429 maize including recommendations on weed resistance management practices. Growers purchasing products containing MON 87429 maize are required by Monsanto's Technology Stewardship Agreement (TSA) to read and follow the TUG. For weed management during the production of hybrid seeds using MON 87429 as the female inbred line, seed producers are encouraged to follow good management practices for herbicide resistance management to minimize the potential for further selection of herbicide-resistant weeds. Furthermore, Monsanto and its affiliates are committed to actively evaluating herbicide performance and weed efficacy on a continuing basis, and developing additional mitigation plans as necessary to manage resistance development for glyphosate, dicamba, glufosinate, quizalofop and 2,4-D.

[9] Weed resistance management guidelines available at http://www.iwm.bayer.com/ and http://www.roundupreadyplus.com/.

VIII.F.5. Weed Management Conclusion

Combining MON 87429 maize with deregulated glyphosate tolerance traits, such as NK603, will allow the use of dicamba, glufosinate, quizalofop, 2,4-D and glyphosate herbicides in an integrated weed management program to control a broad spectrum of grass and broadleaf weed species in maize. These herbicides will also provide distinct sites-of-action for an effective proactive (to delay selection of additional herbicide resistant weeds) and reactive (to manage weed populations that have developed resistance) weed resistance management program in maize. With MON 87429 maize, growers will have options to use and rotate and/or use combinations of herbicides with effective herbicide sites-of-action for in-crop control of glyphosate's hard to control and resistant broadleaf and grass weeds that are present in U.S. maize production.

Furthermore, the integration of MON 87429 maize, along with the glyphosate-tolerant maize trait, will provide growers with the ability to continue use of established maize production practices including tillage systems; the same planting and harvesting machinery; established management of insects, diseases, and other pests; and many of the current herbicides used for weed control, including glyphosate with its established environmental and grower benefits. Therefore, it is not anticipated that the commercialization of MON 87429 maize in the U.S. will have significant impacts on current maize agronomic practices, beyond the intended benefits of more effective and durable management of common and troublesome weeds, including herbicide-resistant weeds and for consistently producing high-quality, high-purity hybrid maize seed.

VIII.G. Crop Rotation Practices in Maize

Crop rotation is a well-established farming practice and a useful management tool for maize production. Crop rotations are used to diversify farm income, spread labor requirements throughout the year, and spread the crop loss risk associated with weather and pest damage across two or more crops. In terms of soil and pest management, rotations are used to 1) manage weed, insect, and disease pests, 2) reduce soil erosion by wind and water, 3) maintain or increase soil organic matter, 4) provide biologically fixed nitrogen when legumes are used in the rotation, and 5) manage excess nutrients (Singer and Bauer, 2009). Studies in U.S. corn belt states indicate maize yield is about 10-15% higher in maize grown following soybean than maize grown following maize (Singer and Bauer, 2009). While there are tangible benefits from crop rotations, many other factors such as crop price fluctuations, input costs, rental agreements, government price supports, weather, choice of farming system and on-farm resources, and other factors all contribute to decisions regarding crop rotations. Table VIII-8 provides an assessment of the common rotational crops following maize at the U.S. country level. Approximately 30% of the U.S. maize acres were rotated back to maize and 57% are rotated to soybean the following year in 2013 (USDA-APHIS Petition #15-113-01p Table VIII-2 pg. 153). Wheat and cotton are other significant rotational crops with approximately 5% and 2%. In addition, based on the integrated pest management annual surveys of a subset of maize growers who planted maize with corn rootworm (CRW) trait in the U.S. in high CRW pressure states of Iowa, Illinois, Indiana, Minnesota, Nebraska and South Dakota, the percentage of continuous maize planting has largely remained the same between 2016, 2017 and 2018 (31%, 27% and 29%, respectively) (ABSTC, 2017; 2018; 2019).

Introduction of MON 87429 maize is not expected to limit crop rotation practices and there would be no significant changes anticipated in post-maize planting due to the availability of MON 87429 maize since (1) the use of dicamba, glufosinate, quizalofop, 2,4-D and glyphosate herbicides does not limit crop rotation choices due to their limited herbicidal persistence and carry-over potential attributes when applied according to the label; and, (2) application use patterns for glufosinate, quizalofop, 2,4-D or glyphosate herbicides for MON 87429 maize will be the same as already approved rates in current biotechnology-derived herbicide tolerant products available to growers and pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of Monsanto's third-generation herbicide tolerance product, MON 87419 maize (U.S. EPA, 2019).

Crop rotation is a valuable tool in managing herbicide resistance. In addition, the use of herbicide mixtures have been shown to be effective in delaying development of herbicide resistance (Beckie and Harker, 2017; Evans et al., 2016). The herbicide tolerance traits in MON 87429 combined with a deregulated glyphosate-tolerance trait support integrated weed management programs that include herbicides and/or herbicide mixtures with diverse sites-of-action, both within a single maize growing season and between maize and rotational crops, to delay the development of herbicide resistance in weeds. Due to natural tolerances or introduced traits, common rotational crops for maize (e.g., soybeans, cotton) may also tolerate dicamba, glufosinate, 2,4-D, quizalofop, and/or glyphosate. However, given the number of sites-of-action for herbicides that may be applied in MON 87429 maize and rotational crops (including those that may be applied for the conventional crops), an herbicide program including sites-of-action that differ between the crops is expected to be feasible.

Table VIII-8. Rotational Crops in the U.S. Following Maize Production

United States Total Maize Acres[1,5]	**Rotational Crops Following Maize**	**Rotational Crop Acres[2,5]**	**% Rotational Crop of Total Maize[2]**
95,365	Maize	28,291	29.7
	Soybean	54,451	57.1
	Wheat	4,527	4.7
	Cotton	1,870	2.0
	Alfalfa[3]	1,303	1.4
	Other Hay	1,118	1.2
	Other crops[4]	3807	3.9
		Total: 95,365	

This table was developed by compiling the data from all four regional summaries.
[1] Maize acreage based on 2013 planting data (USDA-NASS, 2014).
[2]Rotational Crop Acres source: USDA Petition #15-113-01p Table VIII-2 pg. 153
[3] Newly seeded alfalfa.
[4] Other crops: combined acreage of rotated crops following maize that are individually less than 1000,000 acres. It includes sorghum, oats, sugar beets, sunflower, barley, peanut, dry beans, potatoes, tobacco, millet, rice, safflower and vegetables (chili peppers, cantaloupe, watermelon, tomatoes, onions, snap beans, sweet corn, cabbage, lima beans, cucumbers, bell peppers, squash, green peas, carrots).
[5] All acreages are expressed as 1000s of acres. Totals may not be exact due to rounding.

VIII.H. Maize Volunteer Management

Volunteer maize is defined as a maize plant that germinates and emerges unintentionally in a subsequent crop. Volunteer maize commonly occurs in rotational crops in the season following cultivation of maize. Factors reducing the numbers of volunteers in the following crop include the harvesting of maize for silage before grain physiological maturity (approximately 6.1 million U.S. maize acres in 2018 (USDA-NASS, 2019c) and warm, moist climates such as those in the Southeast, where many seeds will germinate in the fall but not survive the winter. In the Northern maize-growing regions, volunteer maize does not always occur in the rotational crop because of seed decomposition over the winter, efficient harvest procedures, and tillage practices.

Management of volunteer maize in rotational crops involves minimizing or reducing the potential for volunteers through practices that include: 1) adjusting harvest equipment to minimize the amount of maize grain lost in the field; 2) planting maize hybrids that reduce the extent of ear drop; 3) choosing maize hybrids with superior stalk strength and reduced lodging; and 4) practicing no-till production to significantly reduce the potential for volunteer growth in the rotational crop. If volunteer maize does occur in subsequent crops, preplant tillage (if significant volunteers have already emerged) and in-crop cultivation are very effective management tools. In addition, Gramoxone® (paraquat) is a preplant option to control volunteer maize, including MON 87429 (Appendix L, Table L-1) alone or with Tricor® (metribuzin), before planting another maize crop (Ikley et al., 2017; Steckel et al., 2009). Similarly, use of Gramoxone® (paraquat) tank mixed with residual herbicides such as metribuzin or linuron is recommended for preplant control of volunteer maize in soybean rotation (Bayer CropScience, 2019a). Several postemergence herbicides also are available to control volunteer maize (conventional or herbicide-tolerant maize) in each of the major maize rotational crops. Because MON 87429 maize is tolerant to the ACCase 'fop' herbicides, quizalofop and other fop herbicides will not be effective to remove volunteer MON 87429 maize. Nonetheless, other ACCase herbicides such as cyclohexanedione or "dims" (e.g., clethodim, sethoxydim) are still effective in control of MON 87429 (Appendix L, Table L-1) as volunteer maize in broadleaf crops. Table VIII-9 provides a summary of labeled selective postemergence herbicides for the effective control of volunteer maize, including MON 87429 maize, in specific rotational crops and include Poast® (sethoxydim), and Select® 2EC (clethodim). These herbicides are labeled for use in 11 vegetable rotation crops and 10 field crops that include soybean, cotton, sugar beet and alfalfa. Imazamox (Raptor®) is an option for postemergence control of volunteer maize in soybean, alfalfa, dry beans, peas, lima bean, snap bean, clover, and edamame (BASF, 2015). In cases of farm sites with grass weed biotypes resistant to quizalofop and/or "dims", and to delay weed resistance development to ACCase herbicides, there are other preplant and postemergence herbicide options available to control grass weeds and/or volunteer maize (see Appendix L, Table L-1). For volunteer control of maize including MON 87429, in wheat crop, there are

® Select is a trademark of Valent U.S.A. Corporation; ® Powerflex, Goldsky and Perfectmatch are the trademarks of Dow AgroSciences Corporation; ® Gramoxone is a trademark of Syngenta Corporation; ® Tricor is a trademark of United Phosphorus, Inc. ® Raptor is a trademark of BASF.

additional postemergence herbicide options available such as Powerflex® (pyroxsulam), GoldSky® (florasulam+ fluroxypyr+ pyroxsulam), Perfectmatch® (clopyralid+ floroxypyr+ pyroxsulam), etc (NDSU, 2018). Additionally, there are also many non-chemical methods of weed control (mechanical removal, tilling, etc.) that are effective in removing unwanted maize, including MON 87429.

MON 87429 combined with deregulated glyphosate tolerance traits, such as NK603, is not expected to notably impact the management of maize volunteer plants based on the availability of various herbicidal and cultivation methods for controlling volunteer MON 87429 maize as well as the demonstrated lack of agronomic and phenotypic differences, including germination and dormancy characteristics, between MON 87429 and conventional maize (see Section VII.C.1).

Table VIII-9. Herbicides Labeled for Control of Volunteer Maize in Labeled Rotational Crops[1]

Crop	Poast	Select 2EC
Soybeans	x	x
Hay	Alfalfa, Clover	Alfalfa, Clover
Cotton	x	x
Sugar Beets	x	x
Sunflower	x	x
Peanuts	x	x
Dry Beans	x	x
Lentils	x	
Potatoes	x	x
Sweet Potatoes	x	x
Vegetables		
Cabbage		x
Cantaloupe		x
Carrots		x
Cucumbers		x
Leaf Lettuce	x	x
Peas, green	x	
Peppers, Chili	x	x
Peppers, Tabasco		x
Onions		Bulbs only
Tomatoes	x	x
Watermelon		x

[1]Source: (BASF, 2019; Valent, 2006)

VIII.I. Stewardship of MON 87429 Maize

Monsanto develops effective products and technologies that deliver value to growers and conserve resources that agriculture depends on and is committed to assuring that its products and technologies are safe and environmentally responsible. Monsanto demonstrates this commitment by implementing product stewardship processes throughout the lifecycle of a product and by participation in the Excellence Through Stewardship® (ETS) Program[10] (BIO, 2012). These policies and practices include rigorous field compliance and quality management systems and verification through auditing. Monsanto's Stewardship Principles are also articulated in Technology Use Guides (Monsanto, 2019) and Monsanto Technology Stewardship Agreements that are signed by growers who utilize Monsanto branded traits, to communicate stewardship requirements and best practices.

As an integral component of fulfilling this stewardship commitment, Monsanto will seek biotechnology regulatory approvals for MON 87429 maize in all important maize import countries with a functioning regulatory system to assure global compliance and support the flow of international trade. These actions will be consistent with the ETS Guide for Product Launch Stewardship of Biotechnology-Derived Plant Products (ETS, 2018), Biotechnology Innovation Organization (BIO) Policy on Product Launches (BIO, 2012). Monsanto continues to monitor other countries that import significant quantities of maize from the U.S., for the development of formal biotechnology approval processes. If new functioning regulatory processes are developed, Monsanto will re-evaluate its stewardship plans and make appropriate modifications to minimize the potential for trade disruption.

Monsanto is also committed following industry best practices on seed quality assurance and control to ensure the purity and integrity of MON 87429 maize hybrid seed. As with all of Monsanto's products, before commercializing MON 87429 maize in any country, a MON 87429 maize detection method will be available via a third-party vendor to maize producers, processors, and buyers regardless of whether they have purchased MON 87429 or not.

Stewardship of glyphosate, dicamba, glufosinate, quizalofop and 2,4-D herbicides to preserve their usefulness to growers is also an important consideration, especially in light of recent U.S. EPA guidance on herbicide resistance management, labeling, education, training and stewardship that is intended to provide growers and registrants strategies to delay herbicide resistance development and prolong efficacy of herbicides (U.S. EPA, 2017b; c). Detailed information regarding dicamba, glufosinate, quizalofop, 2,4-D and glyphosate weed resistance and the usefulness of the herbicide-tolerant MON 87429 maize trait in combination with glyphosate-tolerant traits in maize to address herbicide-resistance issues is presented in Section VIII.F and Appendix K.

[10] www.excellencethroughstewardship.org/

VIII.J. Summary and Conclusion: Impact of the Introduction of MON 87429 Maize on Agricultural Practices

MON 87429 maize has been developed to facilitate greater choices for growers implementing effective weed management including tough-to-control and herbicide resistant broadleaf weeds. The ability to use dicamba, glufosinate, quizalofop and 2,4-D herbicides with multiple sites-of-action can be part of an effective weed management system for maize production in the U.S. Glufosinate, quizalofop, 2,4-D, and glyphosate herbicides are already labeled for use in maize (Liberty®: EPA Reg No. 264-660, Enlist One™: EPA Reg No 62719-695, Assure® II: EPA Reg No. 352-541, Durango® DMA®: EPA Reg No. 62719-556). Pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of Monsanto's third generation herbicide tolerant product MON 87419 maize. For MON 87419 maize, Monsanto requested that EPA allow the 0.5 lb a.e./ac postemergence application window for dicamba to be extended from V5 to V8 growth stage or 36-inch height of maize, whichever occurs first (U.S. EPA, 2019). The combined (pre- and post-emergence) maximum annual application rate of dicamba in MON 87419 maize would be 2.0 lbs. a.e. dicamba per acre per year, increased from the current maximum annual application rate of 0.75 lbs. a.e. in conventional maize. Pending approval of the expanded label at EPA, the dicamba use pattern for MON 87429 would be no different than that of MON 87419. Therefore, the introduction of MON 87429 is not expected to have adverse impacts on current agronomic, cultivation and management practices for commercial grain or hybrid maize seed production. No significant changes are anticipated in crop rotations, tillage practices, planting practices, fertility management, weed and disease management, and volunteer management from the introduction of MON 87429 maize.

IX. PLANT PEST ASSESSMENT

IX.A. Introduction

This section provides a brief review and assessment of the plant pest potential of MON 87429 and its impact on agronomic practices and the environment. USDA-APHIS has responsibility, under the Plant Protection Act (PPA) (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. Regulation 7 CFR § 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article is unlikely to pose a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

According to the PPA, the definition of "plant pest" includes the living stage of any of the following, or a similar article, that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; or (G) an infectious agent or other pathogens (7 U.S.C. § 7702(14)).

The regulatory endpoint under the PPA for biotechnology-derived crop products is not zero risk, but rather a determination that deregulation of the article in question is not expected to pose a potential for plant pest risk. Information in this petition related to plant pest risk characteristics includes: 1) mode-of-action and changes to plant metabolism; 2) composition; 3) expression and characteristics of the gene product; 4) potential for weediness of the regulated article; 5) potential impacts to NTOs; 6) abiotic stressor, disease and pest susceptibilities; 7) impacts on agronomic practices; and 8) impacts on the weediness of any other plant with which it can interbreed, as well as the potential for gene flow. Using the assessment above, the data and analysis presented in this petition lead to a conclusion that MON 87429 is unlikely to pose a plant pest risk, and therefore should no longer be subject to regulation under 7 CFR § 340.

IX.B. Plant Pest Assessment of MON 87429 and Expressed Protein

This section summarizes the details of the genetic insert, characteristics of the genetic modification, and safety and expression of the DMO, PAT, CP4 EPSPS and FT_T proteins expressed in MON 87429 used to evaluate the food, feed, and environmental safety of MON 87429.

IX.B.1. Characteristics of the Genetic Insert and Expressed Protein

IX.B.1.1. Genetic Insert

As described in Section III, MON 87429 was developed by *Agrobacterium*-mediated transformation of maize embryos using plasmid vector PV-ZMHT519224. Characterization of the DNA insert in MON 87429 was conducted using a combination of sequencing, PCR, and bioinformatics methods. The results of this characterization demonstrate that MON 87429 contains one copy of the intended transfer DNA containing the *dmo, pat, ft_t* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple breeding generations. These methods also confirmed that no

vector backbone or other unintended plasmid sequences are present in MON 87429. Additionally, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 87429 to the sequence of the insertion site in conventional maize. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 87429 upon DNA integration.

IX.B.1.2. Mode-of-Action

MON 87429 contains the *dmo* gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide, the *pat* gene from *Streptomyces viridochromogenes* that expresses the phosphinothricin N-acetyltransferase (PAT) protein to confer tolerance to glufosinate herbicide, the *ft_t* gene derived from *Sphingobium herbicidovorans* that expresses a FOPs and 2,4-D dioxygenase (FT_T) protein to confer tolerance to quizalofop and 2,4-D herbicides, and the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 that expresses the CP4 EPSPS protein and contains an siRNA Target Sequence in the 3' UTR, to confer tissue-specific glyphosate tolerance, to facilitate the production of hybrid maize seed.

DMO is an enzyme that catalyzes the demethylation of dicamba to the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA), a well-known metabolite of dicamba in conventional cotton, soybean, livestock and soil (FAO-WHO, 2011b; a; U.S. EPA, 2009). PAT protein acetylates the free amino group of glufosinate to produce non-herbicidal N-acetyl glufosinate, a well-known metabolite in glufosinate-tolerant plants (OECD, 2002a). FT_T protein catalyzes a dioxygenase reaction, in the presence of alpha-ketoglutarate and oxygen, metabolizing quizalofop into quizalofop-phenol and pyruvate and metabolizing 2,4-D into 2,4-dichlorophenol (2,4-DCP) and glyoxylic acid. The safety of 2,4-D, quizalofop, and their metabolites have been assessed by US EPA which concluded that there is a reasonable certainty that no harm will result to the general population, or to infants and children from aggregate exposure to 2,4-D (U.S. EPA, 2017a) or quizalofop (U.S. EPA, 2018), residues or their metabolites. In plants, the endogenous EPSPS protein, an enzyme involved in the shikimate pathway for the biosynthesis of aromatic amino acids, is inhibited by the herbicide glyphosate resulting in cell death (Franz et al., 1997). MON 87429 produces the same CP4 EPSPS protein that is produced in commercial Roundup Ready® crop products which is insensitive to glyphosate and therefore confers tolerance to glyphosate. As described in Section IV.G expression of CP4 EPSPS in MON 87429 plants is driven by the CaMV 35S promoter, a known constitutive promoter (Holtorf et al., 1995; Terada and Shimamoto, 1990). In maize and other monocot plants, CaMV 35S has been shown to drive weak gene expression in pollen tissue (Hamilton et al., 1992; Heck et al., 2005). Additionally, MON 87429 maize utilizes an endogenous maize regulatory element to target CP4 EPSPS mRNA for degradation specifically in tassel tissues, resulting in reduced CP4 EPSPS protein expression in pollen. Appropriately timed glyphosate applications produce a non-viable pollen phenotype and allow for desirable cross pollinations to be made in maize without using traditional methods to control self-pollination in female inbred parents. A focused study on the MON 87429 siRNA Target Sequence demonstrated the absence of unintended effects on endogenous gene regulation. These data taken together with data demonstrating a lack of change in pollen morphology or viability between MON 87429 and control (Section VII) suggest the siRNA Target Sequence for tissue-selective CP4 EPSPS expression does not result in unintended effect on gene regulation in male tissue.

IX.B.1.3. Protein Safety and Expression Levels

The safety and expression of the DMO, PAT, CP4 EPSPS and FT_T proteins are detailed in Section V. MON 87429 DMO, PAT, CP4 EPSPS and FT_T protein levels in forage, root, leaf and grain of MON 87429 were determined using a multiplexed immunoassay. To further demonstrate the MON 87429 RHS trait mode-of-action (MOA), CP4 EPSPS expression in pollen tissue was determined to illustrate the differential expression between vegetative and pollen tissue. The mean DMO protein level in MON 87429 across all sites was highest in leaf at 35 µg/g dw and lowest in root at 2.3 µg/g dw. The mean PAT protein level in MON 87429 across all sites was highest in leaf at 5.8 µg/g dw and lowest in grain at 0.84 µg/g dw. The mean CP4 EPSPS protein level in MON 87429 across all sites was the highest in leaf at 54 µg/g dw and lowest in grain at 0.63 µg/g dw. As intended the mean CP4 EPSPS protein level in MON 87429 pollen across all sites was below the limit of quantitation (0.11 µg/g dw). The mean FT_T protein level in MON 87429 across all sites was the highest in leaf at 440 µg/g dw and lowest in root at 41 µg/g dw.

MON 87429 DMO is an enzyme that catalyzes the demethylation of dicamba to the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) and formaldehyde. DMO proteins have been previously characterized and the safety of these proteins have been well established. Therefore, the consumption of the DMO protein from MON 87429 or its progeny is considered safe for humans and animals.

PAT protein is an acetyltransferase that catalyzes the acetylation of the herbicide glufosinate. PAT proteins, including the PAT protein isolated from MON 87429, have been previously characterized and the safety of the PAT protein has been extensively assessed and in 1997 a tolerance exemption was issued for the PAT protein by U.S. EPA (40 CFR § 180.1151). Therefore, the consumption of the PAT protein from MON 87429 or its progeny is considered safe for humans and animals.

The CP4 EPSPS is key enzyme involved in aromatic amino acid biosynthesis and catalyzes the reaction where the enolpyruvyl group from phosphoenol pyruvate (PEP) is transferred to the 5-hydroxyl of shikimate-3-phosphate to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate. CP4 EPSPS proteins, including the CP4 EPSPS protein from MON 87429, have been previously characterized and the safety of the CP4 EPSPS protein has been extensively assessed. U.S. EPA has established an exemption from the requirement of a tolerance for residues of CP4 EPSPS protein and the genetic material necessary for its production in all plants (U.S. EPA, 1996b). Therefore, the consumption of the CP4 EPSPS protein from MON 87429 or its progeny is considered safe for humans and animals.

The FT_T protein catalyzes a dioxygenase reaction in the presence of αKG and oxygen to metabolize quizalofop, a FOP herbicide, into the herbicidally-inactive quizalofop phenol and pyruvate. The FT_T protein also catalyzes the dioxygenase reaction that degrades 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin herbicide, into herbicidally-inactive 2,4-dichlorophenol (2,4-DCP) and glyoxylic acid in the presence of alpha-ketoglutarate and oxygen. The physicochemical characteristics of the MON 87429-produced FT_T protein were determined and equivalence between MON 87429-produced FT_T and *E. coli*-produced FT_T proteins was demonstrated. Therefore *E. coli*-produced FT_T is an appropriate test substance for

the protein safety studies (heat susceptibility and digestibility). Expression studies demonstrated that FT_T is expressed at low levels in grain. An assessment of the allergenic and toxic potential of the FT_T protein found that the FT_T protein does not pose a significant allergenic risk and was not similar to know toxins. In addition, the donor organism for the MON 87429 FT_T coding sequence is from a genus, *Sphingobium,* that is ubiquitous in the environment and is not commonly known for allergenicity and human or animal pathogenicity. The FT_T protein is rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) and demonstrates no acute oral toxicity in mice at the level tested. Based on the above information, the consumption of the FT_T protein from MON 87429 or its progeny is considered safe for humans and animals. Taken together, the results of these analyses support a determination that MON 87429 is no more likely to pose a plant pest risk than conventional maize.

IX.B.2. Compositional Characteristics

For MON 87429, the introduced proteins, DMO, PAT, CP4 EPSPS and FT_T, confer herbicide tolerance and lack catalytic activity that is intended to or expected to affect the plant's metabolism. Given the nature of these introduced traits and the overall lack of meaningful unintended compositional characteristics observed for biotechnology-derived products characterized to date (Herman and Price, 2013; Venkatesh et al., 2015), compositional changes that would affect the levels of components in MON 87429 maize were not expected. Monsanto is currently in consultation with the Food and Drug Administration following their policy, "Foods Derived from New Plant Varieties," on the food and feed safety of MON 87429 maize (Submitted 05-Feb-2019). Composition data for 25 components including major nutrients in grain (protein, amino acids, total fat, linoleic acid, carbohydrates, acid detergent fiber, neutral detergent fiber and ash), major nutrients in forage (protein, total fat, carbohydrates, acid detergent fiber, neutral detergent fiber and ash) and anti-nutrients in grain (phytic acid and raffinose) were submitted to FDA as part of the voluntary food/feed safety and nutritional assessment for MON 87429 maize. The results of the compositional assessment found that MON 87429 did not meaningfully alter component levels in maize and confirms the compositional equivalence of MON 87429 to conventional maize.

IX.B.3. Phenotypic, Agronomic, and Environmental Interaction Characteristics

An extensive set of comparative plant characterization data were used to assess whether the introduction of dicamba-, glufosinate-, quizalofop- and 2,4-D-tolerance and RHS traits altered the plant pest potential of MON 87429 compared to the conventional control (Section VII). Phenotypic, agronomic, and environmental interaction characteristics of MON 87429 were evaluated and compared to those of the conventional control. As described previously, these assessments included seed germination and dormancy characteristics and pollen characteristics in the laboratory as well as phenotypic and agronomic characteristics and plant responses to abiotic stressors, diseases, and arthropod pests in the field. Results from all phenotypic, agronomic, and environmental interaction assessments demonstrated that MON 87429 does not possess increased weedy characteristics or increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests compared to the conventional control. Taken together, the assessments support a determination that MON 87429 is no more likely to pose a plant pest risk than conventional maize.

IX.B.3.1. Seed Germination and Dormancy

A comparative assessment of seed germination and dormancy characteristics was conducted on MON 87429 and the conventional control. The results of this assessment, including the lack of significant differences and particularly the lack of increased hard seed, support the conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize.

IX.B.3.2. Phenotypic and Agronomic Assessment

Evaluations of phenotypic and agronomic characteristics in the field are useful for assessing characteristics typically associated with weediness, such as lodging and seed loss. The characteristics early stand count, days to flowering, plant height, days to maturity, lodging, seed loss, final stand count, moisture, seed weight, and yield were assessed. The lack of observed differences between MON 87429 and the conventional control for phenotypic and agronomic characteristics across sites supports the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize.

IX.B.3.3. Response to Abiotic Stressors, Diseases, and Arthropod Pests

In an assessment of plant response to abiotic stressors, diseases, and arthropod pests, no differences were observed between MON 87429 and the conventional control for any of the 96 observations for each stressor category across eight sites (Section VII.C.2.2.). The lack of observed differences between MON 87429 and the conventional control for plant responses to abiotic stressors, diseases, and arthropod pests in multiple field environments supports the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased plant pest risk of MON 87429 compared to conventional maize.

IX.B.3.4. Pollen Viability and Morphology

Evaluations of pollen viability and morphology from field-grown plants provide useful information in a plant pest assessment as it relates to the potential for gene flow to, and possible introgression of a biotechnology-derived trait into sexually-compatible plants and wild relatives. No statistically significant differences were detected between MON 87429 and the conventional control for percentage viable pollen or pollen diameter. Furthermore, no visual differences in general pollen morphology were observed between MON 87429 and the conventional control. The lack of observed differences between MON 87429 and the conventional control for pollen characteristics support the overall conclusion that the introduction of the dicamba-, glufosinate-, quizalofop-, and 2,4-D-tolerance and RHS traits does not result in increased weediness or plant pest risk of MON 87429 compared to conventional maize.

IX.B.3.5. Interactions with Non-Target Organisms Including Those Beneficial to Agriculture or Threatened and Endangered Species

The potential for MON 87429 to influence non-target organisms (NTOs) was evaluated using a combination of biochemical information and experimental data. The modes-of-action are well-established and lack toxic effects; experimental evidence reviewed in the current or previously submitted Petitions indicates that the DMO, PAT, CP4 EPSPS and FT_T proteins are safe; and no safety concerns have occurred during the history of environmental exposure to DMO, PAT, CP4 EPSPS and the alpha-ketoglutarate-dependent dioxygenase family of proteins (the class of enzymes to which FT_T belongs). Additionally, molecular characterization did not suggest potential for unintended effects, no evidence was found for unintended changes to plant metabolism, compositional characteristics were substantially equivalent for MON 87429 and the conventional control, and the germination and dormancy, phenotypic and agronomic, and pollen characteristics were not indicative of increased weediness or plant pest risk for MON 87429 compared to conventional maize. Since MON 87429 does not possess pesticidal activity, all organisms that interact with MON 87429 are considered to be NTOs. Environmental interactions data showed a lack of differences in plant responses to a subset of NTOs (specific diseases and arthropod pests). Taken together, these results suggest that MON 87429 is unlikely to adversely affect NTOs, including those beneficial to agriculture, or pose an additional risk to threatened or endangered species or their designated critical habitat above those posed by the cultivation of conventional maize.

According to USDA-APHIS (2014), "Corn possesses few of the characteristics of successful weeds, and has been cultivated around the globe without any report that it is a serious weed or that it forms persistent feral populations." USDA-APHIS (2014) also concluded that states where maize is grown have no plants listed as threatened or endangered, or that are proposed for listing, that are sexually compatible with maize. Because MON 87429 has been shown to be agronomically and phenotypically equivalent to conventional maize without increased weediness potential, the planting of MON 87429 is not expected to affect listed threatened or endangered plant species or designated critical habitat for listed plant or animal species.

The potential for maize to be a host plant for a threatened or endangered species (required by a listed species to complete a portion of its lifecycle) has also been considered. USDA-APHIS (2014) indicates that none of the listed species in states where maize is grown require maize as a host plant. Furthermore, according to USDA-APHIS (2014) and U.S. EPA (2014a; b), there are only a limited number of threatened or endangered species that may be found in maize fields, and there is an even more limited number of species that might feed on maize plants or maize grain. The safety of the MON 87429 DMO, PAT, CP4 EPSPS and FT_T proteins, and the compositional, agronomic and phenotypic equivalence of MON 87429 to conventional maize, support a conclusion that no effects to listed threatened or endangered species, including no biologically significant changes to habitat or diet, are expected due to the planting of MON 87429.

IX.C. Weediness Potential of MON 87429

Although grown extensively throughout the world, maize is not considered a threat to invade natural or agricultural ecosystems. Maize does not establish self-sustaining populations outside of cultivation (Crawley et al., 2001; OECD, 2003; Raybould et al., 2012). This lack of weediness may reflect its poor competitive ability (Olson and Sander, 1988), lack of seed dormancy, and barriers to seed dispersal, as maize cobs retain seed and are covered in a husk (Wilkes, 1972). A number of other characteristics common in weeds, such as rapid flowering following emergence, are lacking in maize (Keeler, 1989). Traits often associated with weediness are typically not selected for during domestication and subsequent breeding and selection. Similarly, the history of maize breeding and production in the U.S. does not indicate there are any changes in the characteristics of maize that would increase the weediness of the crop. Even if kernels of maize are distributed within a field or along transportation routes from the fields to storage or processing facilities, self-sustaining volunteer maize populations are not found growing in fields, fence rows, ditches, or road sides.

In comparative studies between MON 87429 and a conventional control, germination and dormancy, phenotypic and agronomic, environmental interaction, and pollen characteristics were evaluated (Section VII) for changes that would impact the plant pest potential, and in particular, plant weediness potential. Results of these evaluations show that there is no biologically meaningful difference between MON 87429 and the conventional control for characteristics (percentage viable hard seed, lodging, and seed loss) typically associated with weediness. Furthermore, field observations of MON 87429 and the conventional control indicated no differences in plant responses to abiotic stressors, diseases, or arthropod pests. Collectively, these findings support the conclusion that MON 87429 is no more likely to become a weed than conventional maize.

IX.D. Potential for Pollen Mediated Gene Flow and Introgression

Pollen-mediated gene flow (cross pollination) is the first step towards introgression which is the transfer of one or more genes from one plant population to another. Pollen-mediated gene flow and introgression are natural biological processes and do not constitute inherent environmental risks. Gene introgression must be considered in the context of the trait in the biotechnology-derived plant and the likelihood that the presence of the trait and its subsequent transfer to recipient plants will result in increased plant pest potential. The potential for pollen-mediated gene flow from MON 87429 to other cultivated maize and the potential for introgression of the MON 87429 trait to species that can outcross with maize are discussed below.

IX.D.1. Hybridization with Cultivated Maize

Maize is a wind pollinated species with plant morphology that facilitates cross pollination. Therefore, relatively high levels of pollen-mediated gene flow can occur in this species at short distances (Jones and Brooks, 1950). Some biotic and abiotic factors that may influence the amount of pollen-mediated gene flow in maize include: (1) wind direction and speed; (2) distance between the pollen-source and pollen-recipient plants; (3) environmental factors that may impact pollen viability and dispersal (e.g. temperature and relative humidity); (4) duration of pollen shed and (5) floral synchrony between pollen donor and pollen recipient.

The results from several studies conducted on the extent of pollen-mediated gene flow between maize fields demonstrate consistent trends regardless of the experimental design, world region, or detection method. The amount of pollen-mediated gene flow is greatest within the first few meters and decreases sharply with increasing distance from the pollen source (Table IX-1). The distance >200 m (660 feet) is used for managing gene flow during breeding, seed production, identity preservation or other applications; in addition, it forms the basis for the USDA-APHIS performance standards for maize. All U.S. testing and production of regulated MON 87429 seed or grain have been conducted under USDA notification according to these standards. Since no meaningful differences were observed for MON 87429 compared to conventional maize in nutritional value, composition, or in pest/weed potential in field evaluations, no adverse effects are expected from gene flow from commercial production of MON 87429 to other maize.

Table IX-1. Summary of Published Literature on Maize Cross Pollination

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
~1 25 75 126 201 302 402 503	28.6 14.2 5.8 2.3 1.2 0.5 0.2 0.2	Three- year study with one site per year. Outcrossing was detected by yellow seeds in the white-seeded pollen recipient. Values are averaged over years.	USA	(Jones and Brooks, 1950)
1 5 10 14 19 24 28 33 36	19.0 2.6 2.0 0.6 0.4 0.3 0.5 0.3 0.1	Three-year study with two to three sites per year. Outcrossing was detected by yellow seeds in the white-seeded pollen recipient Values are averaged over site-years for the downwind direction.	Canada	(Ma et al., 2004)
24–32 60–62 123–125 244–254 486–500 743–745	0.69 0.23 0.08 0.02 0.005 0.002	Two-year study with two sites per year. Outcrossing was detected by purple seeds in the yellow-seeded pollen recipient. Values are the greatest observed between years for the site where outcrossing was most prevalent.	USA	(Halsey et al., 2005)
1 10 35 100 150 200 250	29.9 2.5 0.4 0.05 0.03 0.03 0.03	Two-year study with one site per year. Outcrossing was detected by yellow seeds in the non-transgenic white-seeded pollen recipient and confirmed by tests for a glyphosate tolerance gene in the yellow seeds. Values are the greatest between years as averaged across all directions from the pollen source.	USA	(Goggi et al., 2006)

Table IX-1. Summary of Published Literature on Maize Cross Pollination (continued).

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
0 2 5 10 20 40 80	12.65 8.81 2.33 3.69 1.05 0.74 0.65	One-year study with one site. Outcrossing was detected by yellow seeds in the white-seeded pollen recipient. Values are the greatest reported at listed distances from the edge of two sampling areas, one adjacent to and one 10 m from the pollen source. The values are half of those observed to reflect flow of a hemizygous gene.	Spain	(Pla et al., 2006)
0 2 5 10 20 25 50 100 150	0.74 0.66 0.35 0.27 0.16 0.14 0.12 0.10 0.12	Three-year study with a total of 55 sites. Outcrossing was quantified on ground samples of nontransgenic pollen recipient grain using a PCR assay for the *pat* herbicide tolerance gene. Values are means for percentage GM DNA for distances with >30 samples.	UK	(Weekes et al., 2007)

Table IX-1. Summary of Published Literature on Maize Cross Pollination (continued).

Pollinator Distance (m)	Reported Outcrossing (%)	Comments	Country	Reference
1 5 10 15 30 60 100 150 200 300	44 16 10 5 1 1 0 0 0 0	Two-year study with one site per year. Outcrossing was detected by herbicide tolerance and PCR tests for an herbicide tolerance transgene in progeny of the non-transgenic pollen recipient. Values are the greatest between years as averaged across all directions from the pollen source.	China	(Zhang et al., 2011)
1 2 4 8 12 16 20 25	12.9 4.6 2.7 1.4 1.0 0.8 0.5 0.5	Three-year study with a total of eight sites. Outcrossing was detected by yellow seeds in the white-seeded pollen recipient. Values are averaged over all directions and sites.	Mexico	(Baltazar et al., 2015)

IX.D.2. Hybridization with Teosinte

For gene flow to occur by typical sexual transmission, the following conditions must exist: (1) the two parents must be sexually compatible; (2) there must be flowering synchrony between the pollen source and pollen recipient; (3) the plants must be within sufficient proximity to each other; and (4) suitable environmental factors, such as relative humidity, temperature, or wind, must be present.

Maize is sexually compatible with certain species or subspecies of teosinte. Although teosinte is not native to the U.S., a compatible subspecies of annual teosinte (*Zea mays* subsp. *mexicana*) is reported to have feral populations in Florida, Alabama, and Maryland (USDA-NRCS, 2019).

Hybrids of maize and teosinte are not expected in the U.S. In a study of maize and *Zea mays* subsp. *mexicana*, very few ovules (approximately 1-2%) produced seed after hand pollination with pollen from the other subspecies (Baltazar et al., 2005). Natural hybridization rates for maize and *Zea mays* subsp. *mexicana* have also been reported to be low (≤0.2% of progeny) (Ellstrand et al., 2007). A genetic barrier to hybridization of maize and teosinte has been described (Evans and Kermicle, 2001) and if present may limit hybridization rates of maize and *Zea mays* subsp. *mexicana*. In addition, differences in developmental and morphological factors (Baltazar et al., 2005), potential differences in flowering time, and limited geographical distribution of teosinte make natural crosses and gene introgression from maize into teosinte unlikely in the U.S.

IX.D.3. Hybridization with *Tripsacum*

Tripsacum is a genus with 15 recognized species (Zuloaga et al., 2003). There are three species of *Tripsacum* that are native to the U.S.: *T. floridanum* (Florida gamagrass), *T. lanceolatum* (Mexican gamagrass), and *T. dactyloides* (Eastern gamagrass) (USDA-NRCS, 2019). Two additional species have introduced populations: *T. latifolium* (wideleaf gamagrass) and *T. fasciculatum* (Guatemalan gamagrass) (USDA-NRCS, 2019).

Tripsacum floridanum (Florida gamagrass) naturally grows in the extreme southern Florida counties of Miami-Dade, Collier, Martin, and Monroe (USDA-NRCS, 2019; Wunderlin et al., 2019). Florida gamagrass has been described as rare and occurring in "low, rocky pinelands" (Blakey et al., 2007) and is categorized as a threatened species in Florida (USDA-NRCS, 2019). *Tripsacum lanceolatum* (Mexican gamagrass) has been reported in Arizona and New Mexico (USDA-NRCS, 2019) and is found on "stream banks or moist cliffs" (de Wet and Harlan, 1978). *Tripsacum dactyloides* (eastern gamagrass) is found primarily throughout the eastern U.S. It is categorized as endangered in Massachusetts and Pennsylvania, threatened in New York, and special concern in Rhode Island (USDA-NRCS, 2019). *Tripsacum fasciculatum* (Guatemalan gamagrass) and *T. latifolium* (wideleaf gamagrass) and are present as introduced populations in Puerto Rico (USDA-NRCS, 2019).

To our knowledge, hybrids between maize and *Tripsacum* do not occur in nature. The formation of hybrids between maize and *Tripsacum* species requires human intervention under specific controlled laboratory conditions, and the hybrids exhibit male sterility that is not resolved by several backcrosses to maize (Russell and Hallauer, 1980). Empirical data showed that "no evidence of gene flow from transgenic maize to eastern gamagrass in nature was observed even

though the two species have grown in close proximity for years and have had ample opportunities for outcrossing" (Lee et al., 2017). Thus, no species of *Tripsacum* is expected to form viable hybrid progeny with maize under natural conditions.

In summary, although hybrids between maize and *Tripsacum* have been produced using specialized laboratory techniques, there is no evidence of hybrid existence in nature. Therefore, gene flow from maize to any *Tripsacum* species is extremely unlikely. Under natural conditions, as is the case with conventional maize, pollen-mediated gene flow from MON 87429 to any species of *Tripsacum* is not expected.

IX.D.4. Transfer of Genetic Information to Species with which Maize Cannot Interbreed (Horizontal Gene Flow)

Monsanto is unaware of any reports regarding the unaided transfer of genetic material from maize species to other sexually-incompatible plant species. The likelihood for horizontal gene flow to occur is exceedingly small. Therefore, potential ecological risk associated with horizontal gene flow from MON 87429 due to the presence of the dicamba, glufosinate, quizalofop and 2,4-D-tolerance and RHS traits are not expected. The consequence of horizontal gene flow of the MON 87429 traits into other plants that are sexually-incompatible is negligible since, as data presented in this petition confirm, the genes and traits confer no increased plant pest potential to maize. Thus, in the highly unlikely event that horizontal gene transfer was to occur, the presence of the MON 87429 traits would not be expected to increase pest potential in the recipient species.

IX.E. Potential Impact on Maize Agronomic Practices

An assessment of current maize agronomic practices was conducted to determine whether the cultivation of MON 87429 has the potential to impact current maize management practices (Section VIII). Maize fields are typically highly managed agricultural areas that are dedicated to crop production. Other than the specific insertion of the *dmo, pat, ft_t and cp4 epsps* coding sequence that provides tolerance to dicamba, glufosinate, quizalofop and 2,4-D, and glyphosate herbicides, MON 87429 is similar to other conventional maize hybrids.

The data presented demonstrate that MON 87429 is similar to conventional maize in its phenotypic and agronomic characteristics, and has levels of susceptibility to abiotic stressors, diseases, and arthropod pests comparable to other conventional maize. Based on this assessment, the introduction of MON 87429 is not likely to impact current U.S. maize agronomic or cultivation practices or lead to an increased plant pest potential compared to other maize hybrids widely available to growers.

IX.F. Conventional Breeding with Other Biotechnology-derived or Conventional Maize

Several biotechnology-derived maize products have been deregulated or are under consideration for deregulation. Once deregulated, MON 87429 may be bred with these deregulated biotechnology-derived maize products, as well as with conventional maize, creating new improved hybrids. APHIS has determined that none of the individual biotechnology-derived maize products it has previously deregulated displays increased plant pest characteristics. APHIS has also concluded that any progeny derived from crosses of these deregulated

biotechnology-derived maize products with conventional or previously deregulated biotechnology-derived maize are unlikely to exhibit new plant pest properties. This presumption, that combined-trait biotechnology products are unlikely to exhibit new characteristics that would pose new plant pest risks or potential environmental impacts not observed in the single event biotech product, is based upon several facts. Namely: 1) stability of the genetic inserts is confirmed in each approved biotech-derived maize product across multiple generations (See Section IV.E for MON 87429 data); 2) stability of each of the introduced traits is continually and repeatedly assessed as new combined-trait hybrids are created by plant breeders and tested over multiple seasons prior to commercialization; 3) combined-trait products are developed using the well-established process of conventional breeding that has been safely used for thousands of years to generate new varieties (Cellini et al., 2004; NRC, 2004; WHO, 1995); 4) worldwide organizations, such as World Health Organization, Food and Agriculture Organization/World Health Organization, International Seed Federation, CropLife International and U.S. FDA, conclude that the safety of the combined-trait product can be based on the safety of the parental GE events (CLI, 2005; FAO-WHO, 1996; ISF, 2005; U.S. FDA, 2001; WHO, 1995); and 5) practical applications in the field have shown that two unrelated biotechnology traits combined together by conventional breeding do not display new characteristics or properties distinct from those present in the single event biotech products (Brookes and Barfoot, 2012; James, 2010; Lemaux, 2008; Pilacinski et al., 2011; Sankula, 2006).

Therefore, based on the considerations above and the conclusion that MON 87429 is no more likely to pose a plant pest risk than conventional maize it can be concluded that any progeny derived from crosses between MON 87429 and conventional maize or other maize with deregulated biotechnology-derived events are no more likely to pose a plant risk than conventional maize.

IX.G. Summary of Plant Pest Assessments

A plant pest, as defined in the Plant Protection Act, is the living stage of any of the following that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; (G) an infectious agent or other pathogens; or (H) any article similar to or allied with any of the articles specified in the preceding subparagraphs (7 U.S.C. § 7702(14)). Characterization data presented in Sections III through VII of this petition confirm that MON 87429, with the exception of the dicamba, glufosinate, quizalofop and 2,4-D-tolerance and RHS traits, is not meaningfully different from conventional maize in terms of plant pest potential. Monsanto is not aware of any other study results or observations associated with MON 87429 that would suggest an increased plant pest risk would result from its introduction.

The plant pest assessment was based on multiple lines of evidence developed from a detailed characterization of MON 87429 compared to conventional maize, followed by a risk assessment on detected differences. The plant pest risk assessment in this petition was based on the following lines of evidence: 1) insertion of a single functional copy of the *pat, dmo, ft_t* and *cp4 epsps* cassette; 2) characterization and safety of the expressed products; 3) compositional equivalence of MON 87429 grain and forage compared to a conventional control; 4) phenotypic, agronomic, and environmental interactions characteristics demonstrating no increased plant pest potential compared to conventional maize; 5) negligible risk to NTOs; 6) familiarity with maize

as a cultivated crop and 7) no greater likelihood to impact agronomic practices, cultivation practices, or the management of weeds, diseases and pests, than conventional maize.

Based on the data and information presented in this petition, it is concluded that, like conventional maize and previously deregulated biotechnology-derived maize, MON 87429 is unlikely to pose a plant pest risk. Therefore, Monsanto Company requests a determination from USDA-APHIS that MON 87429 and any progeny derived from crosses between MON 87429 and other commercial maize be granted nonregulated status under 7 CFR part 340.

X. ADVERSE CONSEQUENCES OF INTRODUCTION

Monsanto knows of no study results or observations associated with MON 87429 indicating that there would be adverse consequences from its introduction. MON 87429 produces the DMO, PAT, CP4 EPSPS and FT_T proteins. The safety of the MON 87429 DMO, PAT and CP4 EPSPS proteins, which have been fully characterized, has been thoroughly assessed in this and previous submissions. The safety of the MON 87429 FT_T protein, which has been fully characterized, has been thoroughly assessed in this submission. As demonstrated by field results and laboratory tests, the only phenotypic differences between MON 87429 and conventional maize are dicamba, glufosinate, quizalofop and 2,4-D tolerance and tissue-specific glyphosate tolerance to facilitate the production of hybrid maize seed.

The data and information presented in this request demonstrate that MON 87429 is unlikely to pose an increased plant pest risk compared to conventional maize. This conclusion is reached based on multiple lines of evidence developed from a detailed characterization of the product compared to conventional maize, followed by risk assessment on detected differences. The characterization evaluations included molecular analyses, which confirmed the insertion of one copy of the intended DNA containing the *pat, dmo, ft_t* and *cp4 epsps* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations.

Analysis of key nutrients of MON 87429 demonstrate that MON 87429 is compositionally equivalent to conventional maize. The phenotypic evaluations, including an assessment of seed germination and dormancy characteristics, phenotypic and agronomic characteristics, pollen characteristics, environmental interactions also indicated MON 87429 is unchanged compared to conventional maize. There is no indication that MON 87429 would have an adverse impact on beneficial or non-target organisms (NTOs), including threatened or endangered species. Therefore, based on the lack of increased pest potential compared to conventional maize, the risks for humans, animals, and other NTOs from MON 87429 are negligible.

The introduction of MON 87429 will not adversely impact cultivation practices or the management of weeds, diseases, and pests in maize production systems. Farmers familiar with commercial maize hybrids will be advised to continue to employ crop rotational practices, weed control practices and/or volunteer control measures that consider the presence of the herbicide tolerance traits while providing the desired agronomic practice(s).

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APPENDICES

Appendix A: USDA Notifications and Permits

Field trials of MON 87429 have been conducted in the U.S. since 2014. The protocols for these trials include field performance, breeding and observation, agronomics, and generation of field materials and data necessary for this petition. In addition to the MON 87429 phenotypic assessment data, observational data on pest and disease stressors were collected from these product development trials. The majority of the final reports have been submitted to the USDA. However, some final reports, mainly from the 2017-2018 seasons, are still in preparation. A list of trials conducted under USDA notifications or permits and the status of the final reports for these trials are provided in Table A-1.

Table A-1. USDA Notifications and Permits Approved for MON 87429 and Status of Trials Planted under These Notifications

Field Trial Year	USDA No.	Effective Date	Trial Status	Release State(s)	Sites
2014	14-080-103n	4/20/2014	Submitted	HI	1
				PR	1
	14-120-113rm	9/2/2014	Submitted	HI	1
2015	14-120-113rm	9/2/2014	Submitted	PR	1
	14-302-112rm	3/1/2015	Submitted	HI	1
				PR	1
	14-309-102rm	3/15/2015	Submitted	IL	5
				IN	1
				KS	5
				NE	4
	14-309-108rm	3/15/2015	Submitted	IA	9
				IL	2
				NE	1

Table A-1. USDA Notifications and Permits Approved for MON 87429 and Status of Trials Planted under These Notifications (continued)

2015	15-042-103n	3/13/2015	Submitted	IA	1
	15-120-112rm	9/1/2015	Submitted	HI	1
2016	15-120-112rm	9/1/2015	Submitted	HI	1
				PR	1
	15-289-112rm	3/2/2016	Submitted	HI	2
	15-306-101rm	3/15/2016	Submitted	IA	6
				IL	2
				KS	4
	15-306-107rm	3/15/2016	Submitted	IA	3
				IL	6
				NE	3
	16-047-104n	3/17/2016	Submitted	IA	1
	16-104-104n	5/11/2016	Submitted	NE	1
	16-116-104n	5/25/2016	Submitted	HI	1
				PR	1
	16-117-104rm	9/1/2016	Submitted	HI	1
				PR	1
	16-117-111rm	9/1/2016	Submitted	HI	1
	16-117-112rm	9/1/2016	Submitted	HI	1
	16-299-102n	11/24/2016	Submitted	HI	1
2017	16-117-111rm	9/1/2016	Submitted	PR	1
	16-182-104rm	12/1/2016	Submitted	HI	1
	16-299-102n	11/24/2016	Submitted	HI	2

Table A-1. USDA Notifications and Permits Approved for MON 87429 and Status of Trials Planted under These Notifications (continued)

2017	16-302-110rm	3/1/2017	Submitted	HI	1
	16-302-111rm	3/1/2017	Submitted	HI	1
	16-315-101rm	3/15/2017	Submitted	IA	5
				IL	10
	16-315-105rm	3/15/2017	Submitted	NE	3
	17-037-101n	3/9/2017	Submitted	CA	1
	17-052-103n	3/23/2017	Submitted	IA	2
				IN	1
				MO	1
				NC	1
				NE	1
				OH	1
				PA	1
	17-053-103n	3/18/2017	Submitted	IA	2
				IL	3
				NE	1
	17-053-105n	3/24/2017	Submitted	IA	1
				IL	2
	17-065-103n	4/5/2017	Submitted	IL	1
	17-100-104n	6/1/2017	Submitted	HI	2
	17-115-111rm	9/1/2017	Submitted	HI	1
2018	17-214-101rm	12/1/2017	Submitted	HI	2

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Table A-1. USDA Notifications and Permits Approved for MON 87429 and Status of Trials Planted under These Notifications (continued)

2018	17-304-101n	11/30/2017	Submitted	HI	3
	17-304-101rm	3/1/2018	In Progress	HI	1
	17-320-101rm	3/15/2018	In Progress	IA	3
				IL	5
				NE	2
	17-320-102rm	3/15/2018	In Progress	IL	2
				NE	1
	18-031-104rm	5/25/2018	In Progress	HI	1
	18-060-107n	3/31/2018	In Progress	IL	2
	18-065-105n	4/5/2018	In Progress	CA	1
				IA	2
				IL	2
				KS	1
				NE	1
				TX	1
				WI	1
	18-085-101n	4/25/2018	In Progress	HI	2
	18-122-103rm	9/1/2018	In Progress	HI	1
	18-122-104rm	9/1/2018	In Progress	HI	1
	18-122-105rm	9/1/2018	In Progress	HI	1
	18-122-106rm	9/1/2018	In Progress	HI	2
	18-283-103n	11/7/2018	In Progress	IL	1
	18-290-101n	11/12/2018	In Progress	IA	1
Total					**158**

Appendix B: Overview, Materials, Methods and Supplementary Results for Molecular Analyses of MON 87429

B.1. Test Substance

The test substance in this study was MON 87429. Five breeding generations of MON 87429 were used to assess the stability of the T-DNA insert. Genomic DNA for use in this study was extracted from seed tissue listed in the table below.

MON 87429 Breeding Generation	Seed ID
R3	11464932
R3F1	11464934
R4	11464931
R4F1	11465707
R5	11465738

B.2. Control Substance

The control substance is the conventional crop variety which is the similar genetic background as the test substances. Genomic DNA was extracted from seed as listed in the table below.

Control Substance	Seed ID	Breeding Generations
LH244	11464930	R3, R4, R5
LH244 × HCL617	11464933	R3F1
LH244 × L7126Z	11465706	R4F1

B.3. Reference Substance

The reference substance was plasmid vector PV-ZMHT519224, which was used to develop MON 87429. Whole plasmid DNA and its sequence served as a positive control for sequencing and bioinformatic analyses. The identity of the reference plasmid was confirmed by sequencing within the study. Documentation of the confirmation of the plasmid vector identity was archived with the raw data. Appropriate molecular weight markers from commercial sources were used for size estimations on agarose gels. The unique identity of the molecular weight markers was documented in the raw data.

B.4. Characterization of Test, Control, and Reference Substances

The Study Director reviewed the chain-of-custody forms to confirm the identity of the test and control substances prior to the use of these materials in the study. Further confirmation of test and control substances identity was determined by sequencing in the study. Test, control and reference DNA and sequencing library substances were considered stable during storage if they yield interpretable signals in sequencing experiments, or did not appear visibly degraded on the stained gels (DNA) or sufficient cluster amplification was observed during flow cell generation (sequencing libraries).

B.5. Genomic DNA Isolation

For sequencing library construction and PCR reactions, genomic DNA was isolated from seed tissues of the test and control substances. First the seeds were decontaminated by vigorously agitating them by hand for 30 seconds with 0.05% (v/v) Tween-20, followed by a tap water rinse. The seeds were then vigorously agitated with 0.5% (w/v) NaOCl, allowed to stand for one minute at room temperature, and rinsed with tap water. The seeds were then vigorously agitated with 1% (v/v) HCl, allowed to stand for one minute at room temperature, and rinsed with tap water. The 1% (v/v) HCl rinse was repeated one time, and then the seeds were rinsed with distilled water and air dried. The dried seeds were ground to a fine powder in a Harbil paint shaker. Genomic DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) extraction protocol. Briefly, 16 ml CTAB buffer (1.5% (w/v) CTAB, 75 mM Tris HCl (pH 8.0), 100 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 1.05 M NaCl, and 0.75% (w/v) PVP) and RNase A was added to ground seed tissue. The samples were incubated at 60°C-70°C for 20-25 minutes with intermittent mixing. The samples were cooled to room temperature and subjected to multiple rounds of chloroform:isoamyl alcohol (24:1) extraction. An additional round of extraction with 10% CTAB solution (10% (w/v) CTAB and 0.7 M NaCl) and chloroform:isoamyl alcohol (24:1) was performed. Genomic DNA was precipitated by adding ~3× volumes of CTAB precipitation buffer (1% (w/v) CTAB, 50 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0) to the samples, followed by resuspension in high salt TE buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 M NaCl). Genomic DNA was precipitated again with 3 M sodium acetate (pH 5.2) and 100% (v/v) ethanol, washed with 70% ethanol, air dried and resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). All extracted DNA was stored in a 4°C refrigerator.

B.6. DNA Quantification

PV-ZMHT519224 DNA and extracted genomic DNA were quantified using a Nanodrop™ Spectrophotometer (Thermo Scientific).

B.7. Agarose Gel Electrophoresis

After quantification, the extracted DNA for NGS library construction was run on a 1% (w/v) agarose gel to check the quality.

B.8. Shearing of DNA

Approximately 1 µg of DNA from the test, control and reference substances were sheared by sonication. The DNA was diluted to ~20 ng/µl in Buffer EB (Qiagen Inc.) and fragmented using the following settings to create approximately 325 bp fragments with 3′ or 5′ overhangs: duty cycle of 10; peak incident power of 175; intensity of 5.0, 200 bursts per cycle, in the frequency sweeping mode at ~3-10°C for 80 seconds.

B.9. Paired End Library Preparation

Paired end genomic DNA libraries were prepared for the test, control, and reference substances using the KAPA Hyper Prep kit (Kapa Biosystems) and a Sage Science BluePippin DNA Size Selection system (Sage Science Inc.) was used to size select the DNA fragments.

First, the 3' and 5' overhangs of the DNA fragments generated by the shearing process were converted into blunt ends and adenylated by following the manufacturer's KAPA Hyper Prep kit instructions (Kapa Biosystems).

Next, adaptors were ligated to the end repaired and A-tailing reaction products by following the manufacturer's KAPA Hyper Prep kit instructions. Following adaptor ligation, an AMPure XP (Beckman Coulter) cleanup was performed on the libraries which were then resuspended in 25 µl of Buffer EB prior to PCR amplification of the libraries. A five cycle PCR amplification of the libraries was carried out following the manufacturer's KAPA Hyper Prep kit instructions. A second AMPure XP cleanup was performed on the libraries which were then resuspended in 22.5 µl of Qiagen Elution Buffer (EB) and stored at -20°C.

The libraries were run on the Sage Science BluePippin Size Selection system using 1.5% agarose gel cassettes and following the manufacturer's instructions. After elution of the desired size range (~500 bp) of DNA fragments, the DNA sample in the elution chamber of the cassette was removed and a 1:1 bead cleanup was performed using the 1x KAPA Pure Beads according to the KAPA Hyper Prep kit instructions.

After bead cleanup, an aliquot of the libraries was analyzed using a Fragment Analyzer QC (Advanced Analytical Technologies, Inc.) according to the manufacturer's instructions. Additional, size selection was conducted using Aline DNA Size Selector-I beads to remove fragments under ~400 bp. An aliquot of the Aline DNA size selected libraries was analyzed using a Fragment Analyzer QC according to the manufacturer's instructions. All purified library DNA was stored in a -20°C freezer.

B.10. Next-Generation Sequencing

The library samples described above were sequenced by Monsanto's Sequencing Technologies using Illumina NextSeq technology that produced short sequence reads (~150 bp long). Sufficient numbers of these sequence fragments were obtained to comprehensively cover the entire genomes of the test samples and the conventional control (Kovalic et al., 2012). Furthermore, a transformation plasmid spike was sequenced to >75× to assess method sensitivity through modeling of 1/10th and one full genome equivalent plasmid spike.

B.11. Read Mapping and Junction Identification

High-throughput sequence reads were captured by aligning to the PV-ZMHT519224 transformation plasmid sequence using the read alignment software Bowtie (V2.2.3) (Langmead and Salzberg, 2012) in order to collect all reads that were sourced from the plasmid as well as reads with sequences representing integration points. Captured reads were subsequently mapped using the FASTA (V36.3.6) local alignment program (Pearson, 2000). All software versions were documented in the archived data package and the software versions which were used in this study have been archived.

B.12. Mapping and Junction Detection

Captured reads from both test and control samples were mapped to the complete PV-ZMHT519224 transformation plasmid sequence in order to detect junction sequences using the FASTA (V36.3.6) local alignment program. Reads with partial matches to the transformation plasmid of at least 30 bases and 96.6% or greater identity were also collected as potential junction sequences (Kovalic et al., 2012).

B.12.1. Effective Sequencing Depth Determination

A single copy locus from the native plant genome (*Zea Mays*) *pyruvate decarboxylase* (*pdc3*, GenBank accession AF370006.2) was used to determine the actual sequence depth coverage. All reads with at least 30 bases matching and 96.6% identity were considered as reads sourced from this locus. Read alignments were further filtered to identify the best alignment as judged by the numerically lowest FASTA alignment *E*-score. Furthermore, final sequencing depths were calculated using a reads best aligning position when multiple alignments were returned. In instances where a read yielded two alignments with identical *E*-scores, both were retained.

Table B-1. Sequencing (NGS) Conducted for the Control and Test Substance

Sample	Total Nucleotide Number (Gb)	Effective Median Depth of Coverage (×-fold)
LH244	286.44	121×
LH244 × HCL617	267.68	106×
LH244 × L7126Z	215.17	86×
MON 87429 (R3)	265.77	108×
MON 87429 (R3F1)	244.53	98×
MON 87429 (R4)	292.95	123×
MON 87429 (R4F1)	269.65	102×
MON 87429 (R5)	254.53	102×

For each sample, the raw data produced are presented in terms of total nucleotide number. Effective depth of coverage is determined by mapping and alignment of all raw data to a single copy locus within the maize genome (*pdc3*). The median effective depth of coverage is shown for all samples.

B.12.2. Positive Control

To produce the positive control sample for sequencing, a plasmid DNA library was created as described in Appendix Sections B.8 to B.10 and spiked into a LH244 conventional control library at approximately 0.03%. The collected data were scaled to represent a single genome equivalent dataset and a $1/10^{th}$ genome equivalent dataset.

Table B-2. Summary of NGS Data for the Conventional Control DNA Sample Spiked with PV-ZMHT519224

	0.1 Genome Equivalent (14× coverage)	1 Genome Equivalent (123× coverage)
Extent of coverage[1] of PV-ZMHT519224	100%	100%
Percent identity of coverage[2] of PV-ZMHT519224	100%	100%

[1]Extent of coverage is calculated as the percent of the PV-ZMHT519224 plasmid that is observed to have coverage by the captured sequence reads.
[2]Percent identity of coverage is calculated as the per position consensus relative to reference of mapped PV-ZMHT519224 bases from the randomly sampled reads.

B.13. PCR and DNA Sequence Analyses to Examine the Insert and Flanking Sequences in MON 87429

Overlapping PCR products, denoted as Product A and Product B were generated that span the insert and adjacent 5' and 3' flanking DNA sequences in MON 87429. For each fragment generation, experimental conditions were chosen to successfully produce on-target amplifications. These products were analyzed to determine the nucleotide sequence of the insert in MON 87429, as well as that of the DNA flanking the 5′ and 3′ ends of the insert, as depicted in Figure IV-7.

The PCR analyses for both Product A and Product B were conducted using approximately 100 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.2 µM of each primer, 0.2 mM of each dNTP, and 1.25 units/reaction of PrimeSTAR GXL Polymerase (TaKaRa Bio Inc.).

The PCR amplification of both Product A and Product B was performed under the following cycling conditions: 25 cycles at 98°C for 10 seconds; 60°C for 15 seconds; 68°C for 9 minutes.

Aliquots of each PCR product were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected sizes. Each PCR product was purified with Agencourt AMPure XP (Beckman Coulter Life Sciences). Approximately 200 ng of purified amplicon was submitted to Monsanto's Genome Sequencing Center for library preparation and MiSeq sequencing.

A consensus sequence was generated by compiling captured reads from MiSeq sequencing of the overlapping PCR products. This consensus sequence was aligned to the PV-ZMHT519224 sequence to determine the integrity and organization of the integrated DNA and the 5' and 3' insert to flank DNA junctions in MON 87429.

B.14. PCR and DNA Sequence Analyses to Examine the Integrity of the DNA Insertion Site in MON 87429

To examine the MON 87429 T-DNA insertion site in LH244 conventional maize, PCR and sequence analyses were performed on genomic DNA from the LH244 conventional control as depicted in Figure IV-7.

The primers used in this analysis were designed from the DNA sequences flanking the insert in MON 87429. A forward primer specific to the DNA sequence flanking the 5' end of the insert was paired with a reverse complement primer specific to the DNA sequence flanking the 3' end of the insert.

The PCR reactions were conducted using approximately 100 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.2 µM of each primer, 0.2 mM of each dNTP, and 1.25 units/reaction of PrimeSTAR GXL Polymerase. The PCR amplification was performed under the following cycling conditions: 30 cycles at 98°C for 10 seconds; 60°C for 15 seconds; 68°C for 30 seconds.

Aliquots of each PCR product were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected sizes. Each PCR product was purified with Agencourt AMPure XP (Beckman Coulter Life Sciences). Approximately 200 ng of purified amplicon was submitted to Monsanto's Genome Sequencing Center for library preparation and MiSeq sequencing.

A consensus sequence was generated by compiling sequences from MiSeq sequencing reactions of the verified PCR product. This consensus sequence was aligned to the 5' and 3' sequences flanking the MON 87429 insert to determine the integrity and any rearrangement of the insertion site.

References for Appendix B

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Langmead, B. and S.L. Salzberg. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9:357-359.

Pearson, W.R. 2000. Flexible sequence similarity searching with the FASTA3 program package. Methods in Molecular Biology 132:185-219.

Appendix C: MON 87429 RHS Mechanism of Action

C.1. Lack of Secondary siRNA from CP4 EPSPS mRNA Suppression Materials and Methods

C.1.1. Low molecular weight RNA blotting

Total RNA from each sample, a 1kb RNA Ladder (Thermo Fisher Scientific) combined with the 25-nt CP4 EPSPS RNA oligos, or the size markers were mixed with the RNA loading Buffer (Ambion, Grand Island, NY), denatured at 95°C for 5 min followed by 4°C for 5 min, loaded to wells of a Criterion Precast 15% polyacrylamide TBE-Urea gel, separated by electrophoresis in a Criterion Cell, and transferred onto positively charged nylon membrane via electroblotting with a Criterion Blotter (Bio-Rad, Hercules, CA) following the manufacture's instruction.

Using T-DNA templates and three sets of the gene-specific primers (Table C-1), three fragments covering the entire CP4 EPSPS transcript except the siRNA target sequence (to avoid cross hybridization with the endogenous mts-siRNAs) (Figure C-1) were obtained by PCR amplifications using Ex Taq DNA polymerase (Takara Bio Inc.) and purified with the QIAquick Gel Extraction Kit (Qiagen). DIG-labeled probes CP4 EPSPS 5', CP4 EPSPS 3', and OsGRP3 3'UTR were prepared from the PCR products with a PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland). A 25-nt RNA oligo with a sequence (GGGUGCUGACAUCGAGGUUAUCAAU) identical to a portion of the CP4 EPSPS mRNA for use as a positive control and two DIG-labeled 20- and 25-nt RNA oligos for use as size markers were synthesized. Hybridizations were then conducted at 38°C with rotation in DIG Easy Hyb (Sigma). Post-hybridization washes were done as recommended by the manufacture, and DIG detection was performed using reagents and Lumi-Film X-ray films from Roche. In addition, the RNA blot was rinsed in RNase-free water briefly, incubated in the Stripping Buffer (0.2% SDS and 0.1× SSC) for 45 min at 70°C twice to remove the DIG-labeled probes, equilibrated in 2× SSC, and then reprobed with a synthetic DIG-labeled 21-nt DNA oligo (DIG-CAGAGCTCCCTTCAATCCAAA) designed for detection of zm-miR159b as a control.

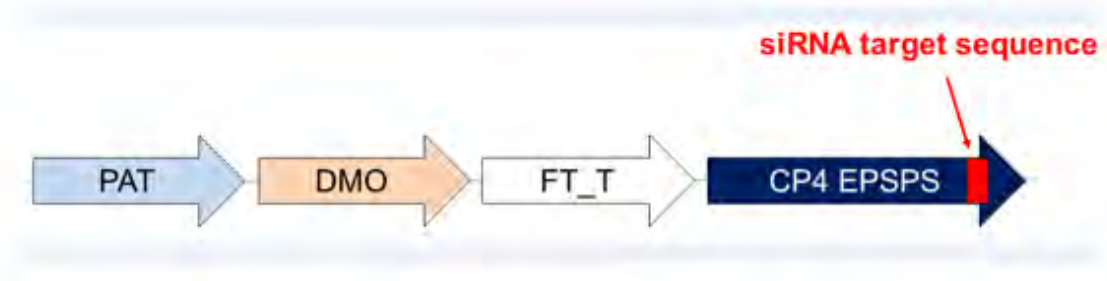


Figure C-1. Transgenic elements in the T-DNA for generating plants containing MON 87429

PAT: phosphinothricin N-acetyltransferase; DMO: dicamba mono-oxygenase; FT_T: FOPs and 2,4-D tolerance enzyme variant T (α-ketoglutarate-dependent dioxygenase); CP4 EPSPS: *Agrobacterium sp.* strain CP4 5-enolpyruvylshikimate-3-phosphate synthase; siRNA TS: small interfering RNA Target Sequence. Not drawn to scale.

C.2. Expression of Endogenous Genes with Sequence Homology to the siRNA Target Sequence Materials and Methods

Total RNA was used for expression analyses of endogenous genes by a TaqMan or a SYBR Green assay (Table C-1). One-step real-time PCR was performed in singleplex with three replicates for each RNA sample. Maize elongation factor 1a (EF1a) was used as a normalizer. Relative expression levels were determined as described in Yang et al 2009.

Table C-1. Primers and probes used in this study

Primer ID	Sequence	Tm	Expected product
For expression analyses of endogenous genes			
Forward Primer 1	AAACCTACGGGAAAGTTGCG	61.33	105 bp
FAM Probe 1	6FAM-ACAAGAAATACCCCTGCTCCAACCTG-MGBNFQ	68.10	
Reverse Primer 1	TCTCGCATAATTTGTAGTTCAGCTG	61.83	
Forward Primer 2	TGGATGAATGKAATCTGAACAAGCT	62.13	73 bp
Reverse Primer 2	CGGGGCAMTTGAACAATCCTA	62.97	
For normalization of expression			
EF1a Forward Primer	GCTAGCTTTACCTCCCAGGTCATC	63.55	64 bp
EF1a VIC Probe	VIC-TCATGAACCACCCTGGC-MGBNFQ	73.29	
EF1a Reverse Primer	GGGCATAGCCATTGCCAATC	64.68	

Table C-1. Primers and probes used in this study (continued)

For preparation of probes for Northern hybridization			
CP4 EPSPS 5' Probe F	AGCAGCATCCACGAGCTTAT	59.98	709 bp
CP4 EPSPS 5' Probe R	ATGGGTTCAATCACGGTTGT	60.08	
CP4 EPSPS 3' Probe F	GCGCTAATCTAACGGTCGAA	60.33	737 bp
CP4 EPSPS 3' Probe R	TCAAGCGGCCTTAGTATCAGA	59.96	
OsGRP3 3'UTR Probe F	CATCGTGGCCAGTTATCCTT	59.93	554 bp
OsGRP3 3'UTR Probe R	TGCAAAATGGAAATGCTGTG	60.63	

References for Appendix C

Yang H, Schmuke JJ, Flagg LM, Roberts JK, Allen EM, Ivashuta S, et al. A novel real-time polymerase chain reaction method for high throughput quantification of small regulatory RNAs. Plant Biotechnol J. 2009; 7: 621–630. pmid:1961

Appendix D: Characterization of FT_T Protein Produced in MON 87429

D.1. Characterization of the FT_T Protein

D.1.1. FT_T Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties and confirmation of the safety of the introduced protein(s). For the safety data generated using the *E. coli*-produced FT_T protein to be applied to the MON 87429-produced FT_T protein (plant-produced FT_T), the equivalence of the plant- and *E. coli*-produced proteins must first be demonstrated. To assess the equivalence between the MON 87429-produced FT_T and *E. coli*-produced FT_T proteins, a small quantity of the MON 87429-produced FT_T protein was purified from MON 87429 grain. The MON 87429-produced FT_T protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 87429-produced and *E. coli*-produced FT_T proteins was assessed using a panel of analytical tests; as shown in Table D-1. Taken together, these data provide a detailed characterization of the MON 87429-produced FT_T protein and establish the equivalence of the MON 87429-produced FT_T and *E. coli*-produced FT_T proteins. Based on this established equivalence, conclusions derived from digestibility, heat susceptibility and oral acute toxicology studies conducted with *E. coli*-produced FT_T protein are applicable to MON 87429-produced FT_T protein.

Table D-1. Summary of MON 87429 FT_T Protein Identity and Equivalence

Analytical Test	Assessment	Analytical Test Outcome
1. N-terminal sequence	Identity	• The expected N-terminal sequence for MON 87429-produced FT_T and was observed by Nano LC-MS/MS[1]
2. Nano LC-MS/MS[1]	Identity	• Nano LC-MS/MS[1] analysis of trypsin digested peptides from for MON 87429-produced FT_T protein yielded peptide masses consistent with expected peptide masses from the theoretical trypsin digest of the amino acid sequence
3. Western blot analysis	Identity and Equivalence	• MON 87429-produced FT_T protein identity was confirmed using a western blot probed with antibodies specific for FT_T protein • Immunoreactive properties of the MON 87429-produced FT_T and the *E. coli*-produced FT_T proteins were shown to be equivalent
4. Apparent molecular weight (MW)	Equivalence	• Electrophoretic mobility and apparent molecular weight of the MON 87429-produced FT_T and the *E. coli*-produced FT_T proteins were shown to be equivalent
5. Glycosylation analysis	Equivalence	• Glycosylation status of MON 87429-produced FT_T and *E. coli*-produced FT_T proteins were shown to be equivalent
6. FT_T enzymatic activity	Equivalence	• Functional activity of the MON 87429-produced FT_T and the *E. coli*-produced FT_T proteins were shown to be equivalent

[1] Nano LC-MS/MS = Nanoscale liquid chromatography-tandem mass spectrometry

The details of the materials and methods for the panel of analytical tests used to evaluate and compare the properties of the MON 87429-produced FT_T and *E. coli*-produced FT_T proteins are described at the end of Appendix D. A summary of the data obtained to support a conclusion of protein equivalence is below.

D.1.2. Results of the N-Terminal Sequencing Analysis

The expected N-terminal sequence for the FT_T protein deduced from the *ft_t* gene present in maize of MON 87429 was confirmed by Nano LC-MS/MS. The experimentally determined sequence corresponds to the deduced FT_T protein beginning at the initial alanine position (Figure D-1). The alanine is derived from the chloroplast transit peptide, MDH (Section V.D.1). Alternative cleavage of CTP from FT_T in *planta* by a general stromal processing peptidase is common (Richter and Lamppa, 1998). The N-terminal sequencing results for MON 87429-produced FT_T protein were consistent with the sequencing results for the *E. coli*-produced FT_T protein (Figure D-1). The expression plasmid designed to express the mature MON 87429 FT_T protein in *E. coli* included an N-terminal methionine residue that was likely cleaved by methionine aminopeptidase and other aminopeptidases. Therefore, the *E. coli*-produced FT_T sequence begins at position 2 of the expected sequence deduced from the *E. coli* expression plasmid, which corresponds to position 1 of the mature FT_T protein present in MON 87429. The cleavage of the N-terminal methionine from proteins *in vivo* by methionine aminopeptidase is common in many organisms (Bradshaw et al., 1998). Hence, the sequence information confirms the identity of the FT_T protein isolated from the grain of MON 87429.

Amino Acids Residue # from the N-terminus	→	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
E. coli-produced FT_T sequence	→	A	M	H	A	A	L	T	P	L	T	N	K	Y	R	F
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Expected FT_T Sequence	→	A	M	H	A	A	L	T	P	L	T	N	K	Y	R	F
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
MON 87429-produced FT_T Experimental Sequence	→	A	M	H	A	A	L	T	P	L	T	N	K	Y	R	F

Figure D-1. N-Terminal Sequence of the MON 87429-Produced FT_T Protein

The experimental sequence obtained from the MON 87429-produced FT_T was compared to the expected sequence of the mature protein deduced from the *ft_t* gene, a modified version of the R-2,4-dichlorophenoxypropionate dioxygenase (*Rdpa*) gene, present in MON 87429. The *E. coli*-produced FT_T sequence begins at position 2 of the expected sequence deduced from the *E. coli* expression plasmid, which corresponds to position 1 of the mature FT_T protein present in MON 87429. The single letter International Union of Pure and Applied Chemistry- International Union of Biochemistry (IUPAC-IUB) amino acid code is A, alanine; M, methionine; H, histidine; L, leucine; T, threonine; P, proline; N, asparagine; K, lysine; Y, tyrosine; R, arginine; F, phelyalanine.

D.1.3. Results of Mass Fingerprint Analysis

Peptide mass fingerprint analysis is a standard technique used for confirming the identity of proteins. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by peptide mapping is considered to be reliable if ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments (Biron et al., 2006; Krause et al., 1999). The identity of the MON 87429-produced FT_T protein was confirmed by Nano LC-MS/MS analysis of peptide fragments produced by the trypsin digestion of the MON 87429-produced FT_T protein.

There were 34 unique peptides identified that corresponded to the masses expected to be produced by trypsin digestion of the MON 87429-produced FT_T protein (Table D-2). The identified masses were used to assemble a coverage map of the entire FT_T protein (Figure D-2A). The experimentally determined coverage of the FT_T protein was 97% (Figure D-2A 288 out of 296 amino acids). This analysis further confirms the identity of MON 87429-produced FT_T protein.

There were 20 unique peptides identified that corresponded to the masses expected to be produced by trypsin digestion of the *E. coli*-produced FT_T protein (Table D-3) by MALDI-TOF MS analysis during the protein characterization. The identified masses were used to assemble a coverage map of the entire FT_T protein (Figure D-2B). The experimentally determined coverage of the *E. coli*-produced FT_T protein was 65% (Figure D-2B, 194 out of 297 amino acids). This analysis further confirms the identity of *E. coli*-produced FT_T protein.

Table D-2. Summary of the Tryptic Masses Identified for the MON 87429-Produced FT_T Using Nano LC-MS/MS

[1]Experimental Mass[2]	Calculated Mass[3]	Diff[4]	Fragment[5]	Sequence[6]
1266.6764	1266.6754	0.001	1 – 12	AMHA...LTNK
1308.6891	1308.686	0.0031	1 - 12	*AMHA…LTNK
2531.3715	2531.3748	-0.0033	13 - 35	YRFI...VDLR
2212.2081	2212.2104	-0.0023	15 - 35	FIDV...VDLR
6659.3313	6659.3289	0.0024	15 - 73	FIDV...AFSR
4465.1336	4465.1291	0.0045	36 - 73	EPLD...AFSR
1336.7871	1336.7867	0.0004	74 - 85	RFGP...PILK
1180.6852	1180.6856	-0.0004	75 - 85	FGPV...PILK
2583.3786	2583.3771	0.0015	75 - 97	FGPV...QMIR
1420.7026	1420.702	0.0006	86 - 97	SIEG...QMIR
947.4422	947.442	0.0002	98 - 105	REANESSR
791.3409	791.3409	0	99 - 105	EANESSR
2560.2049	2560.2057	-0.0008	106 - 128	FIGD...VVMR
4500.1576	4500.1505	0.0071	129 - 170	AIEV...SATK
5922.8591	5922.8549	0.0042	129 - 182	AIEV...TNWR
1440.7149	1440.715	-0.0001	171 - 182	VFGS...TNWR
2204.1021	2204.1015	0.0006	171 - 189	VFGS...TSVK
781.3974	781.397	0.0004	183 - 189	FSNTSVK
3362.7007	3362.7042	-0.0035	183 - 213	FSNT...VTGR
976.4284	976.4284	0	190 - 198	VMDV...AGDR
2599.317	2599.3177	-0.0007	190 - 213	VMDV...VTGR
1640.8988	1640.8999	-0.0011	199 - 213	ETVH...VTGR
1796.9998	1797.001	-0.0012	199 - 214	ETVH...TGRR
1601.7407	1601.7442	-0.0035	214 - 225	RALY...YCQK
1445.6441	1445.6431	0.001	215 - 225	ALYC...YCQK
1078.4969	1078.4965	0.0004	226 - 235	IQGM...AESK
1448.7651	1448.7663	-0.0012	236 - 247	SLLQ...HATK

[1] All imported values were rounded to 4 decimal places.
[2] Only experimental masses that matched calculated masses with the highest scores are listed.
[3] The calculated mass is the relative molecular mass calculated from the matched peptide sequence.
[4] The calculated difference = (experimental mass – calculated mass).
[5] Position refers to amino acid residues within the predicted MON 87429-produced FT_T sequence as depicted in Figure D-1.
[6] For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are shown separated by dots (…).
[*] acetylation and oxidation observed.

Table D-2. Summary of the Tryptic Masses Identified for the MON 87429-Produced FT_T Using Nano LC-MS/MS (Continued)

[1]Experimental Mass[2]	Calculated Mass[3]	Diff[4]	Fragment[5]	Sequence[6]
2275.1126	2275.1096	0.003	236 - 253	SLLQ...FTCR
844.3548	844.3538	0.001	248 - 253	FDFTCR
1913.9253	1913.924	0.0013	258 - 272	KDQV...TMHR
1785.8299	1785.8291	0.0008	259 - 272	DQVL...TMHR
819.4129	819.4127	0.0002	273 - 280	AVPDYAGK
1122.5821	1122.5822	-0.0001	273 - 282	AVPD...GKFR
1030.5409	1030.5407	0.0002	288 - 296	TTVA...KPSR

[1] All imported values were rounded to 4 decimal places.
[2] Only experimental masses that matched calculated masses with the highest scores are listed.
[3] The calculated mass is the relative molecular mass calculated from the matched peptide sequence.
[4] The calculated difference = (experimental mass – calculated mass).
[5] Position refers to amino acid residues within the predicted MON 87429-produced FT_T sequence as depicted in Figure D-1.
[6] For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are shown separated by dots (…).
[*] acetylation and oxidation observed.

Table D-3. Summary of the Tryptic Masses Identified for *E. coli*-produced FT_T Using MALDI-TOF MS[1]

Experimental Mass[2]	Calculated Mass[3]	Diff.[4]	Fragment[5]	Enzyme	Sequence[6]
1266.5457	1266.6754	-0.1297	2 - 13	Trypsin	AMHA…LTNK
2211.9397	2212.2104	-0.2706	16 - 36	Trypsin	FIDV…VDLR
1336.6551	1336.7867	-0.1316	75 - 86	Trypsin	RFGP...PILK
1180.5685	1180.6856	-0.1171	76 - 86	Trypsin	FGPV...PILK
1420.5619	1420.7020	-0.0792	87 - 98	Trypsin	SIEG...QMIR
791.2727	791.3409	-0.0682	99 - 106	Trypsin	REANESSR
2559.8802	2560.2057	-0.3255	107 - 129	Trypsin	FIGD…VVMR
1440.5745	1440.7150	-0.1405	172 - 183	Trypsin	VFGS...TNWR
781.3226	781.3970	-0.0744	184 - 190	Trypsin	FSNTSVK
2598.9771	2599.3177	-0.3406	191 - 214	Trypsin	VMDV…VTGR
1640.7249	1640.8999	-0.1749	200 - 214	Trypsin	ETVH…VTGR
1603.5482	1603.7123	-0.1640	215 - 226	Trypsin	RALY…YCQK
1447.4541	1447.6112	-0.1570	216 - 226	Trypsin	ALYC…YCQK
1448.6154	1448.7663	-0.1509	237 - 248	Trypsin	SLLQ…HATK
2275.8086	2276.0936	-0.2850	237 - 254	Trypsin	SLLQ…FTCR
845.2653	845.3378	0.0725	249 - 254	Trypsin	FDFTCR
1786.6216	1786.8131	-0.1915	260 - 273	Trypsin	DQVL…TMHR
819.3327	819.4127	-0.0799	274 - 281	Trypsin	AVPDYAGK
1030.4390	1030.5407	-0.1017	288 - 297	Trypsin	TTVAGDKPSR

[1] All imported values were rounded to 4 decimal places.
[2] Only experimental masses that matched calculated FT_T trypsin digested masses are listed in the table.
[3] The calculated mass is the exact molecular mass calculated from the matched peptide sequence.
[4] The calculated difference = experimental mass - calculated mass.
[5] Position refers to amino acid residues within the predicted *E. coli*-produced FT_T sequence.
[6] For peptide matches greater than nine amino acids in length, the first 4 residues and last 4 residues are show separated by three dots (...).

(A)

001 AMHAALTPLT NKYRFIDVQP LTGVLGAEIT GVDLREPLDD STWNEILDAF

051 HTYQVIYFPG QAITNEQHIA FSRRFGPVDP VPILKSIEGY PEVQMIRREA

101 NESSRFIGDD WHTDSTFLDA PPAAVVMRAI EVPEYGGDTG FLSMYSAWET

151 LSPTMQATIE GLNVVHSATK VFGSLYQATN WRFSNTSVKV MDVDAGDRET

201 VHPLVVTHPV TGRRALYCNQ VYCQKIQGMT DAESKSLLQF LYEHATKFDF

251 TCRVRWKKDQ VLVWDNLCTM HRAVPDYAGK FRYLTRTTVA GDKPSR

(B)

002 MAMHAALTPL TNKYRFIDVQ PLTGVLGAEI TGVDLREPLD DSTWNEILDA

052 FHTYQVIYFP GQAITNEQHI AFSRRFGPVD PVPILKSIEG YPEVQMIRRE

102 ANESSRFIGD DWHTDSTFLD APPAAVVMRA IEVPEYGGDT GFLSMYSAWE

152 TLSPTMQATI EGLNVVHSAT KVFGSLYQAT NWRFSNTSVK VMDVDAGDRE

202 TVHPLVVTHP VTGRRALYCN QVYCQKIQGM TDAESKSLLQ FLYEHATKFD

252 FTCRVRWKKD QVLVWDNLCT MHRAVPDYAG KFRYLTRTTV AGDKPSR

Figure D-2. Peptide Map of the MON 87429-Produced FT_T and *E. coli*-Produced FT_T

(A) The amino acid sequence of the MON 87429-produced FT_T protein was deduced from the *ft_t* gene, a modified version of the R-2,4-dichlorophenoxypropionate dioxygenase (*Rdpa*) gene, present in MON 87429. Boxed regions correspond to peptides that were identified from the MON 87429-produced FT_T protein sample using Nano LC-MS/MS. In total, 97% coverage (288 out of 296 amino acids) of the expected protein sequence was covered by the identified peptides.

(B) The amino acid sequence of the *E. coli*-produced FT_T protein was deduced from the *ft_t* gene, a modified version of the R-2,4-dichlorophenoxypropionate dioxygenase (*Rdpa*) gene, that is contained on the expression plasmid. Boxed regions correspond to peptides that were identified from the *E. coli*-produced FT_T protein sample using MALDI-TOF MS. In total, 65% coverage (194 out of 297 amino acids) of the expected protein sequence was covered by the identified peptides.

D.1.4. Results of Western Blot Analysis of the FT_T Protein Isolated from the Grain of MON 87429 and Immunoreactivity Comparison to *E. coli*-produced FT_T Protein

Western blot analysis was conducted using mouse anti-FT_T monoclonal antibody to provide additional confirmation of the identity of the FT_T protein isolated from the grain of MON 87429 and to assess the equivalence of the immunoreactivity of the MON 87429-produced and *E. coli*-produced FT_T proteins. The results showed that immunoreactive bands with the same electrophoretic mobility were present in all lanes loaded with the MON 87429-produced and *E. coli*-produced FT_T proteins (Figure D-3). For each amount loaded, comparable signal intensity was observed between the MON 87429-produced and *E. coli*-produced FT_T protein bands. As expected, the signal intensity increased with increasing load amounts of the MON 87429-produced and *E. coli*-produced FT_T proteins, thus, supporting the identity of the MON 87429-produced FT_T protein.

To compare the immunoreactivity of the MON 87429-produced and the *E. coli*-produced FT_T proteins, densitometric analysis was conducted on the bands that migrated at the expected apparent molecular weight (MW) for FT_T proteins (~35 kDa). The signal intensity (reported in OD × mm^2) of the band of interest in lanes loaded with MON 87429-produced and the *E. coli*-produced FT_T proteins was measured (Table D-4). Because the mean signal intensity of the MON 87429-produced FT_T protein band was within 35% of the mean signal of the *E. coli*-produced FT_T protein, the MON 87429-produced FT_T and *E. coli*-produced FT_T proteins were determined to have equivalent immunoreactivity.

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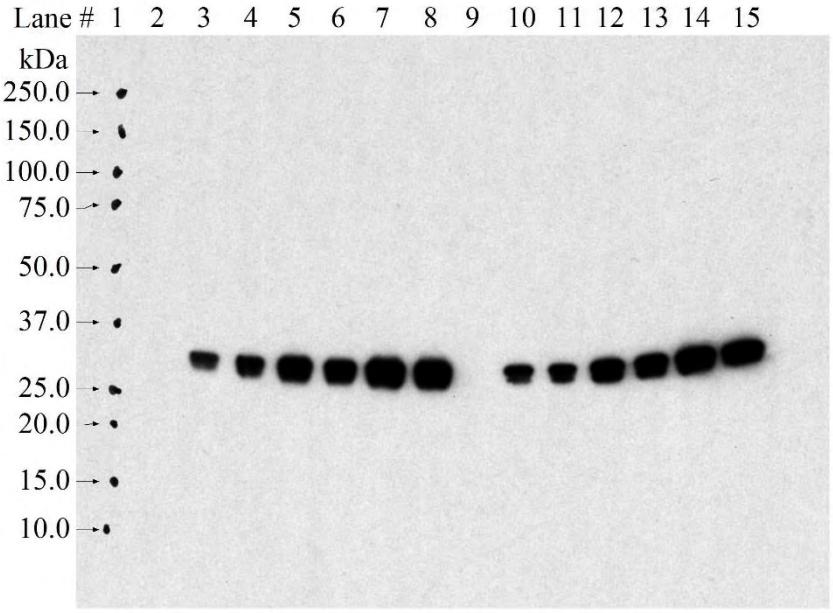


Figure D-3. Western Blot Analysis and Immunoreactivity of MON 87429-Produced and *E. coli*-Produced FT_T Proteins

Aliquots of the MON 87429-produced FT_T protein and the *E. coli*-produced FT_T protein were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. Proteins were detected using mouse anti-FT_T monoclonal antibody and then horse anti-mouse polyclonal antibody conjugated with peroxidase. Immunoreactive bands were visualized using an ECL system. The approximate MW (kDa) of the standards are shown on the left. The 45 second exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Blank	-
3	MON 87429-produced FT_T	5
4	MON 87429-produced FT_T	5
5	MON 87429-produced FT_T	10
6	MON 87429-produced FT_T	10
7	MON 87429-produced FT_T	20
8	MON 87429-produced FT_T	20
9	Blank	-
10	*E. coli*-produced FT_T	5
11	*E. coli*-produced FT_T	5
12	*E. coli*-produced FT_T	10
13	*E. coli*-produced FT_T	10
14	*E. coli*-produced FT_T	20
15	*E. coli*-produced FT_T	20

Table D-4. Immunoreactivity of the MON 87429-Produced and *E. coli*-Produced FT_T Proteins

Mean Signal Intensity from MON 87429-Produced FT_T[1] (OD x mm^2)	Mean Signal Intensity from E. coli-Produced FT_T[1] (OD x mm^2)	Acceptance Limits[2] (OD x mm^2)
94,017.0	97,454.4	63,345.4 – 131,563.4

[1] Each value represents the mean of six values (n = 6).
[2] The acceptance limits are for the MON 87429-produced FT_T protein and are based on the interval between -35% (97,454.4 x 0.65 = 63,345.4) and +35 % (97,454.4 x 1.35 = 131,563.4) of the mean of the *E. coli*-produced FT_T signal intensity across all loads.

D.1.5. Results of the FT_T Protein Molecular Weights and Purity Analysis

For apparent MW and purity determination, the MON 87429-produced FT_T and the *E. coli*-produced FT_T proteins were subjected to SDS-PAGE. Following electrophoresis, the gel was stained with Brilliant Blue G-Colloidal stain and analyzed by densitometry. The MON 87429-produced FT_T protein (Figure D-4, lanes 3-8) migrated with the same mobility on the gel as the *E. coli*-produced FT_T protein (Figure D-4, lane 2) and the apparent MW was calculated to be 36.0 kDa (Table D-5). Because the experimentally determined apparent MW of the MON 87429-produced FT_T protein was within the acceptance limits for equivalence (Table D-6), the MON 87429-produced FT_T and *E. coli*-produced FT_T proteins were determined to have equivalent apparent molecular weights.

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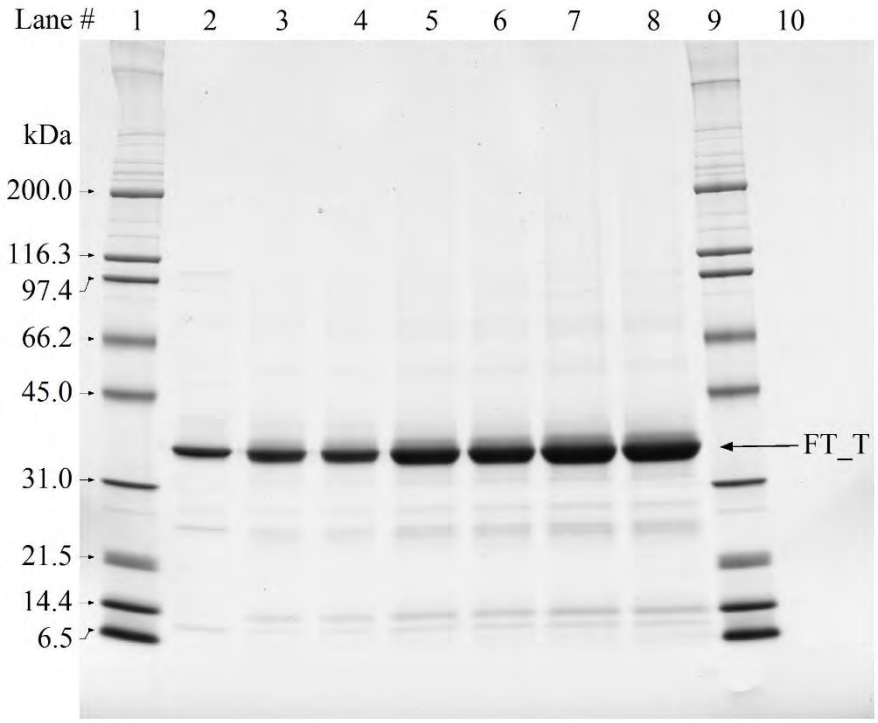


Figure D-4. Purity and Apparent Molecular Weight Analysis of the MON 87429-Produced FT_T Protein

Aliquots of the MON 87429-produced and the *E. coli*-produced FT_T proteins were subjected to SDS-PAGE and the gel was stained with Brilliant Blue G-Colloidal stain. The MWs (kDa) are shown on the left and correspond to the standards loaded in lanes 1 and 9. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standard	5.0
2	*E. coli*-produced FT_T	1.0
3	MON 87429-produced FT_T	1.0
4	MON 87429-produced FT_T	1.0
5	MON 87429-produced FT_T	2.0
6	MON 87429-produced FT_T	2.0
7	MON 87429-produced FT_T	3.0
8	MON 87429-produced FT_T	3.0
9	Broad Range MW Standard	5.0
10	Blank	

Table D-5. Apparent Molecular Weight and Purity Analysis of the MON 87429-Produced FT_T Protein

	Apparent MW[1] (kDa)	Purity[2] (%)
Average (n=6)	36.0	91

[1]Final MW was rounded to one decimal place.
[2]Average % purity was rounded to the nearest whole number.

Table D-6. Apparent Molecular Weight Comparison Between the MON 87429-Produced FT_T and *E. coli*-Produced FT_T Proteins

Apparent MW of MON 87429-Produced FT_T Protein (kDa)	Apparent MW of *E. coli*-Produced FT_T Protein (kDa)	Acceptance Limits[1] (kDa)
36.0	35.5	34.1 – 36.8

[1] Data obtained from the *E. coli*-produced FT_T protein was used to generate a prediction interval for setting the acceptance limits (Table D-8).

D.1.6. FT_T Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether the FT_T protein was glycosylated when expressed in the maize grain of MON 87429, the MON 87429-produced FT_T protein was analyzed using an ECL™ glycoprotein detection method. Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 87429-produced and *E. coli*-produced FT_T proteins, the *E. coli*-produced FT_T protein was also analyzed.

A clear glycosylation signal was observed at the expected molecular weight (~ 80 kDa) in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure D-5, panel A). In contrast, no glycosylation signal was observed in the lanes containing the *E. coli*-produced FT_T protein or MON 87429-produced FT_T protein (Figure D-5, panel A).

To confirm that MON 87429-produced FT_T and *E. coli*-produced FT_T proteins were appropriately loaded for glycosylation analysis, a second membrane with identical loadings and transfer time was stained with Coomassie Blue R250 for protein detection. Both the MON 87429-produced and *E. coli*-produced FT_T proteins were detected (Figure D-5, panel B). These data indicate that the glycosylation status of MON 87429-produced FT_T protein is equivalent to that of the *E. coli*-produced FT_T protein and that neither is glycosylated.

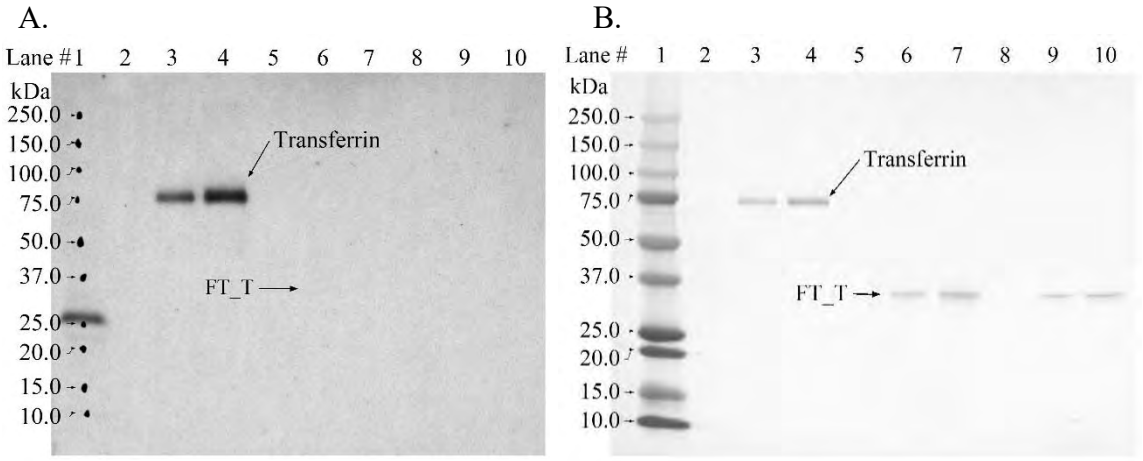


Figure D-5. Glycosylation Analysis of the MON 87429-Produced and *E. coli*-Produced FT_T Proteins

Aliquots of the transferrin (positive control), *E. coli*-produced FT_T and MON 87429-produced FT_T were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. The MWs (kDa) correspond to the Precision Plus Dual Color Protein™ Standards. The arrows show the expected migration of the MON 87429-produced and *E. coli*-produced FT_T proteins and transferrin. (A) Where present, the labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based detection using ECL reagents and exposure to Hyperfilm®. The 30 second exposure is shown. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	-
2	Blank	-
3	Transferrin (positive control)	100
4	Transferrin (positive control)	200
5	Blank	-
6	MON 87429-produced FT_T	100
7	MON 87429-produced FT_T	200
8	Blank	-
9	*E. coli*-produced MON 87429 FT_T	100
10	*E. coli*-produced MON 87429 FT_T	200

D.1.7. FT_T Functional Activity

The functional activities of the MON 87429-produced and *E. coli*-produced FT_T proteins were determined using a colorimetric assay that measures the FT_T catalyzed degradation of 2,4-D to 2,4-DCP. In this assay, protein-specific activity is expressed as units per microgram of protein (U/mg), where a unit (U) is 1 nmole of 2,4-D to 2,4-DCP per min at 25°C. The MON 87429-produced and *E. coli*-produced FT_T proteins were considered to have equivalent functional activity if the specific activity of both were within the preset acceptance limits of 183 to 974 U/mg (the prediction interval calculated from a data set of historically determined FT_T protein activity; see Table D-9).

The experimentally determined specific activity for the MON 87429-produced and *E. coli*-produced FT_T proteins are presented in Table D-7. The specific activities of MON 87429-produced and *E. coli*-produced FT_T proteins were 723 U/mg and 720 U/mg of FT_T protein, respectively. Because the specific activities of MON 87429-produced and *E. coli*-produced FT_T proteins fall within the preset acceptance limits (Table D-7), the MON 87429-produced FT_T protein was considered to have equivalent functional activity to that of the *E. coli*-produced FT_T protein.

Table D-7. Functional Activity of MON 87429-Produced and *E. Coli*-Produced FT_T Protein

MON 87429-Produced FT_T [1] (U/mg)	*E. coli*-Produced FT_T [1] (U/mg)	Acceptance Limits[2] (U/mg)
723	720	183.4 – 974.0

[1] Value refers to mean calculated based on samples spectrophotometrically read in triplicate plate wells.
[2] Data obtained from two *E. coli*-produced FT_T lots were used to generate a prediction interval for setting the acceptance limits (Appendix D, Table D-9)

D.1.8. FT_T Protein Identity and Equivalence Conclusion

The MON 87429-produced FT_T protein was purified from MON 87429 grain, was characterized, and a comparison of the physicochemical and functional properties between the MON 87429-produced and the *E. coli*-produced FT_T proteins was conducted following a panel of analytical tests: 1) N-terminal sequence analysis established the same identity for the MON 87429-produced and *E. coli*-produced FT_T proteins; 2) Nano LC-MS/MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the *ft_t* gene product present in MON 87429; 3) the MON 87429-produced and the *E. coli*-produced FT_T proteins were both detected on a western blot probed with antibodies specific for FT_T protein and the immunoreactive properties of both proteins was shown to be equivalent; 4) the electrophoretic mobility and apparent molecular weight of the MON 87429-produced and *E. coli*-produced FT_T proteins were shown to be equivalent; 5) the glycosylation status of MON 87429-produced and *E. coli*-produced FT_T proteins was determined to be equivalent; and 6) the functional activity of the MON 87429-produced and *E. coli*-produced FT_T was demonstrated to be equivalent. These results demonstrate that the MON 87429-produced FT_T protein and the *E. coli*-produced FT_T protein are equivalent. This demonstration of protein equivalence confirms that the *E. coli*-produced FT_T protein is appropriate for use in the evaluation of the safety of the MON 87429-produced FT_T protein.

D.2. Materials and Methods for Characterization of FT_T Protein Produced in MON 87429

D.2.1. Materials

The test substance is the MON 87429-produced FT_T protein (lot 11478812) purified from grain of MON 87429 (lot 11464934). The MON 87429-produced FT_T protein was stored in a -80 ºC freezer in a buffer containing 50 mM Tris, pH 8, 200 mM NaCl.

The reference substance is the *E. coli*-produced FT_T protein (lot 11478164). The FT_T reference substance was generated from cell paste produced by fermentation of E. coli containing pMON374248 expression plasmid. The DNA sequence encoding FT_T protein contained in the expression plasmid was confirmed both prior to and following fermentation.

D.2.2. Protein Purification

The plant-produced FT_T protein used in this equivalence study was purified from grain of MON 87429. The purification procedure was not performed under a good laboratory practice (GLP) plan; however, all procedures were documented in an electronic lab notebook (ELN) and, where applicable, standard operating procedures (SOPs) were followed. All purification steps were performed at ~4°C, except where specifically stated. FT_T was purified from an extract of ground grain using immunoaffinity chromatography (IAC). A detailed description of the purification procedure specific for FT_T was archived in the Monsanto Regulatory Archives and a brief purification procedure was described below.

Grain of MON 87429 was ground to fine powder in the presence of dry ice and stored at -80°C until use. A total of ~50 g of ground powder was mixed with 0.5 L of Extraction Buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 2 mM benzamidine-HCl) at room temperature for ~1 hr. The slurry

was centrifuged twice at 13,500 x g for 15 min at ~4°C to be clarified, and about 410 ml supernatant was collected, filtered, and loaded onto a ~1 ml IAC column (Column size: ID = 1 cm, h ≈ 1.5 cm; Resin: MabSelect Protein A resin cross-linked with ~3 mg of anti-FT_T mAb (Monsanto, lot 109529) following the manufacturer's instruction Catalog #: 17-5199-03, GE Healthcare, IL) which was connected to a AKTA FPLC system at 4°C. Before sample load, the resin was equilibrated with Equilibration Buffer (50 mM Tris, pH 8.0, 200 mM NaCl). The sample was loaded on the resin at 500 cm/hr. The resin was subsequently washed 50 column volumes (CV) Equilibration Buffer and 35 CV Wash Buffer (50 mM Tris, pH 8.0, 1 M NaCl). The protein was eluted with 50 CV Elution Buffer (2 M arginine, 50 mM Tris, pH 8.0, 1 M NaCl). As the fractions were collected, they were immediately diluted 1:3 with Equilibrium Buffer. Fractions containing FT_T, identified by SDS-PAGE analyses, were pooled to a final volume of ~200 ml. This pooled sample was concentrated and buffer-exchanged to storage buffer (50 mM Tris, pH 8.0, 200 mM NaCl) using centrifugal filtration devices (Millipore, Cat. #: UFC901024). This FT_T protein, purified from the grain of MON 87429, was aliquoted, assigned lot 11478812, and stored in a -80 °C freezer.

D.2.3. Methods for Characterization

D.2.3.1. N-Terminal Sequencing/Mass Fingerprint Analysis

The test substance was analyzed for N-terminal amino acid sequence and peptide mass fingerprint analysis by Nano LC-MS/MS. An aliquot of the test substance was subjected to acetone precipitation to remove buffer components and re-suspended in 30 µl 40% TFE/100 mM NH_4HCO_3. The sample was reduced by 10 mM DTT at 37ºC for 1 hr, alkylated by incubation in the dark for 30 min at room temperature with 50 mM iodoacetamide (IAM), and then trypsin digested in an enzyme to protein ration of 1:20. at 37ºC. After 15 hr the digestion was quenched with 1µl of formic acid. The digest was extracted with 30 µl of 60% acetonitrile/0.1% FA twice. The sample was dried to completion using vacuum centrifugation and then solubilized in 20 µl of 0.1 % FA for LC-MS/MS analysis.

The LC-MS/MS data acquisition was performed on a Dionex 3000 Ultimate nanoLC system (Dionex) interfaced to an Orbitrap Fusion mass spectrometer (Thermo Scientific), which is equipped with a nano-ESI source. The sample was loaded and separated online using an Acclaim PepMap100 C18 nano column (75 µm- id × 150 mm, 2 µm, 100 Å, Dionex). The separation of the digests was achieved at a 300 nl/min flow rate using an acetonitrile gradient as follows: 0-4 min sustaining 2% solvent B (100% acetonitrile with 0.1% FA), 4-80 min ramping solvent B 2-40%, 80-85 min ramping solvent B 40-90%. The column was washed/equilibrated between injections as follows: 85-90 min ramping maintaining solvent B at 90%, 90-90.1 min decreasing solvent B 90-2%, and 90.1-100 min maintaining solvent B at 2%. Solvent A was 0.1% FA.

The Orbitrap Fusion mass spectrometer was operated with two scan events. The first scan event was a full Fourier transform mass spectrometry scan with a range of m/z from 200 to 1800 and a mass resolution of 120,000 at m/z of 200. The second scan event was a tandem mass spectrometry scan of fragments from collision induced dissociation (CID) of precursor ions from the first scan event with an isolation width of 2.0 m/z.

The LC-MS/MS dataset were analyzed using Mascot (version 2.5, Matrix Science). MS/MS spectra were searched against the theoretical FT_T protein sequence. The mass tolerances of MS1 mass and MS/MS mass were set as 5 ppm and 0.5 Da, respectively. Carboxymethylation of cysteine was set as a fixed modification. Methionine oxidation and N-terminal acetylation were set as variable modifications. A maximum of two missed cleavages for trypsin were allowed. The mass coverage was accepted if it is greater than 40% of the protein sequence.

D.2.3.2. Western Blot Analysis-Immunoreactivity

Western blot analysis was performed to confirm the identity of the MON 87429-produced FT_T protein and to compare the immunoreactivity of the MON 87429-produced and *E. coli*-produced FT_T protein. MON 87429-produced and *E. coli*-produced FT_T proteins were initially diluted to 200 ng/µl (purity-corrected FT_T protein concentration) with Milli-Q water, and finally diluted to 2 ng/µl in 5 × LB and Milli-Q water and then heated to 100°C for 5 min. Three amounts (~5, ~10, and ~20 ng) of the intact test substance (total protein concentration × purity of the intact FT_T protein) and the intact reference substance (total protein concentration × purity of the intact FT_T protein) were loaded in duplicate onto a pre-cast Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight standards (Precision Plus Protein Dual Color Standards™, Bio-Rad) were loaded on the gel for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

The Western blotting procedure was performed using an iBind™ Western System apparatus (Life Technologies). The membrane was blocked with 1 × iBind™ Solution (Life Technologies) and incubated with mouse anti-FT_T monoclonal antibody (Monsanto, lot #: 109529) at a dilution of 1:1250 in 1 × iBind™ Solution. The membrane was then washed with 1 × iBind™ Solution and incubated with horse anti-mouse polyclonal antibody conjugated with peroxidase (Vector, Cat #: PI-2000, lot #: X0328) at a dilution of 1:1250 in 1 × iBind™ Solution. After washing with 1 × iBind™ Solution, immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare) and exposed to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta).

Quantification of the bands on the blot was performed on a Bio-Rad GS-900 densitometer with the supplied Image Lab 5.0 Security Edition software using the volume tool. The signal intensities of the immunoreactive bands migrating at the expected position for the FT_T protein were quantified as volume values. The immunoreactivity was reported in OD × mm^2.

D.2.3.3. Apparent Molecular Weight and Purity Determination by SDS-PAGE

MON 87429-produced and *E. coli*-produced FT_T proteins were diluted in 5 × loading buffer (LB, 0.31 M Tris-Cl, pH 7.5, 10% SDS, 50% glycerol, 25% (v/v) 2-mercaptoethanol, 0.025% (w/v) Bromophenol blue) and Milli-Q water and then heated to 95-105°C for 3-5 min. The MON 87429-produced FT_T protein was loaded in duplicate at ~1.0, ~2.0, and ~3.0 µg, based on total protein concentration, onto a Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen) in lanes 3, 4, 5, 6, 7 and 8. The *E. coli*-produced FT_T protein was loaded at ~1.0 µg total protein in lane 2. Broad Range Molecular Weight Standards (Bio-Rad) were prepared and

loaded in lanes 1 and 9 on the gel. Following electrophoresis at a constant voltage, proteins were briefly fixed in 40% (v/v) methanol, 7% (v/v) acetic acid and stained for 18 hr with Brilliant Blue G-Colloidal stain (Sigma). Gels were briefly destained in 10% (v/v) acetic acid, 25% (v/v) methanol followed by 7 hr in 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-900 densitometer supplied with Image Lab 5.0 Security Edition software. Apparent MW and purity were reported as an average of all 6 lanes containing the MON 87429-produced FT_T protein.

D.2.3.4. Glycosylation Analysis

ECL Glycoprotein Detection method (GE Healthcare) was used for glycoprotein detection. The MON 87429-produced FT_T protein, *E. coli*-produced FT_T protein and a positive control (Transferrin, Sigma) were diluted in 5 × LB and water to a final concentration of 25 µg/ml protein and 1 × LB and then heated to 100.0°C for 5 min. Two amounts (~100 ng and ~200 ng) of the intact MON 87429-produced FT_T protein (purity-corrected), the *E. coli*-produced FT_T protein (purity-corrected), and the positive control were loaded onto a pre-cast Tris-glycine 4-20% polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight markers (Precision Plus Protein Dual Color Standards™, Bio-Rad) were also loaded for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

Glycosylation analysis was performed on the PVDF membrane at room temperature using the ECL Glycoprotein Detection Module (GE Healthcare) as directed by the manufacturer. Glycosylated proteins were detected using equivalent chemical reagents to the ECL™ reagents (GE Healthcare) and Amersham Hyperfilm (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta). An identical gel was run and electrotransferred to a PVDF membrane in parallel. Proteins were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) and then destained with 1× Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad). After washing with water, the blot was scanned using Bio-Rad GS-900 densitometer with the supplied Image Lab 5.0 Security Edition software.

D.2.3.5. Functional Activity

In preparation for analysis, the *E. coli*-produced FT_T protein was initially diluted with storage buffer to the same purity-corrected concentration as the MON 87429-produced FT_T protein (0.82 mg FT_T/ml). Prior to functional analysis, both MON 87429-produced and *E. coli*-produced FT_T proteins were diluted with assay reaction buffer (20 mM MOPS, pH 6.8, 50 µM $(NH_4)_2Fe(SO_4)_2$, 50 µM sodium ascorbate, 0.5 mM α-ketoglutarate) to a purity-corrected concentration of ~10 µg/ml. The reactions were performed in assay reaction buffer with 5 µg/ml FT_T at ~25°C for 30 min. The reactions for both proteins were conducted in triplicate and were initiated by the addition of 2,4-D to a final concentration of 0.5 mM. After 30 min incubation, the reactions were quenched with 15 µl stop buffer (50 mM Boric acid, pH 10.0, 50 mM potassium chloride, 1 mM EDTA). A 800 µM 2,4-dichlorolphenol (2,4-DCP) solution was used to prepare a standard curve ranging from 0 to 400 µM 2,4-DCP in assay reaction buffer and then quenched with 15 µl stop buffer. An assay blank (assay reaction buffer only) and negative control (0.5 mM 2,4-D in assay reaction buffer) were also run in triplicate. After quenching the reactions, 15 µl of 0.8% potassium ferricyanide was added to standard, blank, negative control

and sample wells and incubated at ~25°C for 1 min. The absorbance of each reaction and standard was measured at 510 nm with a reference wave length of 700 nm using SpectraMax 384 plus Microplate Reader. The amount of 2,4-DCP in each reaction was determined using the standard curve. The specific activity of FT_T was defined in unit per µg of protein (U/mg), where a unit (U) is 1 nmole of 2,4-D to 2,4-DCP per min at 25°C.

D.2.3.6. Prediction Intervals as Acceptance Criteria

Acceptance criteria (acceptance limits) based on prediction intervals were used to assess the equivalence of the MON 87429-produced and *E. coli*-produced FT_T proteins for apparent MW and functional activity. A prediction interval is an estimate of an interval in which a randomly selected future observation from a population will fall, with a certain degree of confidence, given what has already been observed; *i.e.*, prediction intervals are generated based on statistical analysis of existing data.

The source of the data used to generate the prediction intervals for apparent MW and functional activity for FT_T protein equivalence assessment are provided in Table D-8 and Table D-9, respectively. The data used were generated under GLP guidelines and included apparent MW and functional activity estimates for the reference *E. coli*-produced FT_T protein during the initial characterization and for an additional *E. coli*-produced FT_T protein lot during re-characterization (functional assay only).

The two-sided 95% prediction interval (PI) for one future assay was calculated using JMP software (version 9.00, SAS Institute, Inc., Cary, NC) according to the guidance document of Ramírez (2009).

Table D-8. Individual Assay Data and 95% Prediction Interval Generated for the Apparent MW of FT_T Protein for One Future Assay

Assay Number[1]	Apparent MW of FT_T Protein (kDa)[2]
1	35.833
2	35.589
3	36.033
4	35.989
5	34.827
6	35.006
7	34.690
8	35.729
Mean	35.5
Standard Deviation	0.54
95% Prediction Interval	34.1 – 36.8

[1] Assay 1-8 represents the MW data from the initial characterization of *E. coli*-produced FT_T (lot 11478164).

[2] The values in the table represent the mean of six (n=6) data points within each assay. With 95% confidence, the mean of the 6 data points from the next single assay of the population will fall within the stated interval. Mean and prediction interval values rounded to one decimal point.

Table D-9. Individual Assay Data and 95% Prediction Interval Generated for the Functional Activity of the FT_T Protein for One Future Assay

Assay Number[1]	Functional Activity of FT_T Protein[2] (U/mg)
1	742.671
2	756.702
3	842.367
4	775.289
5	748.196
6	761.855
7	554
8	486
9	293
10	343
11	440
12	410
13	471
14	583
15	473
Mean	578.7
Standard Deviation	178.46
95% Prediction Interval	183.4 – 974.0

[1] Assay 1-6 represents the specific activity from the initial characterization of *E. coli*-produced FT_T (lot 11478164). Assays 7-15 represent the specific activity data for re-characterization of a different lot of *E. coli*-produced FT_T (lot 11462796).

[2] The values in the table represent the mean of 3 replicates (n=3) within each assay. With 95% confidence, the mean of the 3 replicates from the next single assay of the population will fall within the stated interval. Mean and prediction interval values rounded to one decimal point.

References for Appendix D

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Appendix E: Specificity of the MON 87429 Dicamba Mono-Oxygenase (DMO) Enzyme Using o-Anisic Acid as a Substrate

E.1. MON 87429 DMO o-Anisic Acid Substrate Specificity

Dicamba mono-oxygenase (DMO) is an enzyme that catalyzes the demethylation of the herbicide dicamba (3,6-dichloro-2-methoxybenzoic acid) to the non-herbicidal 3,6-dichlorosalycilic acid (DCSA) (Chakraborty et al., 2005). In order to evaluate the selectivity of DMO for dicamba herbicide as compared to potential endogenous substrates, a series of *in vitro* studies were previously performed and reported in MON 87708 USDA-AHPIS Petition #10-188-01p. Endogenous compounds evaluated in those studies included ferulic acid, *o*-anisic acid, sinapic acid, syringic acid, and vanillic acid. These substrates were chosen because they are structurally similar to dicamba and are found in plants. The results demonstrated that DMO did not catabolize the potential endogenous substrates tested and supported the conclusion that DMO has a high specificity for dicamba. The *Escherichia coli* (*E. coli*)-produced DMO used in MON 87708 USDA-AHPIS Petition #10-188-01p had an identical amino acid sequence to the wild-type DMO found in *Stenotrophomonas maltophilia*, with the exception of a histidine-tag at the N-terminus.

MON 87429 maize contains a *dmo* expression cassette that results in two forms of the DMO protein, DMO+0 and DMO+1, collectively referred to herein as MON 87429 DMO. The DMO+0 differs from the wild-type DMO by only a leucine added at position 2 (Wang et al., 2016). The DMO+1 has one additional cysteine on the N-terminus of the DMO+0, which is derived from a chloroplast transit peptide. Due to the close similarity of the DMO protein used in MON 87708 USDA-AHPIS Petition #10-188-01p and MON 87429 DMO, the two enzymes were expected to exhibit the same level of substrate specificity. This was evaluated by testing for potential catabolism of *o*-anisic acid by *E. coli*-produced MON 87429 DMO, using the same qualitative assay reported in MON 87708 USDA-AHPIS Petition #10-188-01p. Although *o*-anisic acid is not known to be present in corn, this substance was chosen for this confirmatory experiment since, among the five substrates used in the MON 87708 USDA-AHPIS Petition #10-188-01p, this is the substrate that is most structurally similar to dicamba. The results were evaluated qualitatively. For *o*-anisic acid, similarly to the previously reported results in MON 87708 USDA-AHPIS Petition #10-188-01p, no new peaks that would be indicative of the predicted demethylated product were observed, confirming that the MON 87429 DMO did not catabolize *o*-anisic acid. These results taken together with the previously reported results demonstrate that DMO has a high specificity for dicamba as a substrate.

E.2. Materials and Methods for MON 87429 DMO o-Anisic Acid Substrate Specificity

MON 87429 produces two forms of the DMO protein, DMO+0 and DMO+1, collectively referred to herein as MON 87429 DMO. The MON 87429 DMO used in the in vitro enzyme assays was co-expressed in *E. coli* using a single expression vector designed to

match the DMO+0 and DMO+1 produced in MON 87429. The DMO+0 has an identical amino acid sequence to the wild-type DMO protein isolated from *S. maltophilia*, with the exception of a leucine added at position 2 (Wang et al., 2016). The DMO+1 has an identical amino acid sequence to the DMO+0, with the exception of one additional cysteine on its N-terminus derived from a chloroplast transit peptide.

The compounds tested and standards used in the in vitro enzyme assays are shown in Table E-1.

Table E-1. Compounds Used in Specificity In Vitro Enzyme Assays

Manufacturer/ Retailer	Compound	Common Name
Compounds Tested:		
Millipore	2-methoxybenzoic acid	*o*-anisic acid
Sigma	3,6-dichloro-2-methoxybenzoic acid	dicamba

E.3. In Vitro Enzymatic Reaction Method

The reaction of the *E. coli*-produced MON 87429 DMO with dicamba or *o*-anisic acid was carried out using the three enzymes necessary for demethylation: 0.077 µg/µL reductase (lot 11406970), 0.02 µg/µL ferredoxin (lot 11423290), and 0.005 µg/µL *E. coli*-produced MON 87429 DMO (lot 11472501), in a reaction mixture containing 25 mM KPi, H_2O, 10 mM $MgCl_2$, 0.7 mM NADH, 0.0004 U/µL formaldehyde dehydrogenase, 0.5 mM FeSO4, and the tested compound at 0.3 mM. Control assays were run by substituting dilution buffer (25 mM KPi, pH 7.2, 10 mM MgCl2) for DMO in equal volume for the above reactions. The final volume for each assay sample was 200 µl. Each assay sample was incubated for 15 min at ~30ºC before quenching the reaction by the addition of 5% H_2SO_4. The activity of DMO with dicamba provided a positive control for DMO functional activity.

E.4. Liquid Chromatography Separation Method

Each reaction mixture was separated by UPLC using an ACQUITY UPLC BEH C18 Column containing 1.7 µm Bridged Ethyl Hybrid (BEH) particles. The column was heated to and maintained at 40°C. The substrates and products formed were detected by ACQUITY UPLC UV detector with optimized wavelengths (dicamba at 226 and 280nm, anisic acid at 210 and 280 nm) (LC-UV). The chromatography was performed at 0.05 ml/min and following the separation the column effluent was then directed to the mass spectrometer. Both mobile phase A (H_2O) and solvent B (acetonitrile) contained 0.1% v/v formic acid. Gradients used were as following:

The gradient was run from 40 to 60% solvent B in 6 min, 60 to 100% solvent B in 4 min and then kept at 100% solvent B for 2 min before returning to 40% solvent B in 0.1 min. DCSA was used as a standard to determine product retention time.

A 5 µl injection of reaction mixture was used to monitor the presence of the tested compound.

E.5. Mass Spectrometry Method

Elution from the UPLC column flowed directly to a Waters Micro Q-TOF mass spectrometer. The parameters used for the mass determination of all compounds were: negative mode, capillary voltage of 2500 V, extraction cone of 1.5 V, source temperature of 80°C, and the desolvation temperature was 120°C. The sample cone voltage was 25 V for dicamba, DCSA, and *o*-anisic acid. The desolvation gas flow was 500 L/hour and scan time was 0.76 seconds and inter scan delay was 0.1 sec. The m/z range used was specific to each tested compound and product. The m/z range scanned for dicamba and DCSA was from 160 to 224 from 0 to 15 min. The m/z at 175, which is a fragment ion of dicamba, was used as the detection ion for dicamba. This fragment ion of dicamba gave better sensitivity than the parent ion. The m/z at 205 was used to detect DCSA. The m/z range scanned for *o*-anisic acid was from 120 to 230 within 15 min. The m/z at 151 was used to detect *o*-anisic acid, and the m/z at 137 was used to look for a new peak that would be indicative of a potential demethylation product of *o*-anisic acid.

E.6. Results and Discussion

The potential catabolism of *o*-anisic acid by *E. coli*-produced MON 87429 DMO was evaluated using the same qualitative enzymatic assay reported in MON 87708 USDA-AHPIS Petition #10-188-01p. The conversion of dicamba to DCSA was also assessed. Mass spectrometry scans from 120 m/z to 230 m/z were analyzed to cover the range of tested compounds and all potential demethylated products formed by the reaction of *o*-anisic acid and *E. coli*-produced MON 87429 DMO. For *o*-anisic acid, no additional peaks that might be associated to the predicted demethylated product were observed in the mass spectrometry scans from 120 m/z to 230 m/z. Figure E-1 shows a representative result from the reaction mixtures containing *o*-anisic acid, with and without *E. coli*-produced MON 87429 DMO. *o*-Anisic acid is shown at its respective detection mass (m/z 151; Figure E-1B). A single demethylation of *o*-anisic acid to produce a compound with m/z 137 was predicted based on the known mode of action of DMO. No new peaks were observed at m/z 137 in the chromatograms (Figure E-1C) for the samples containing DMO as compared to the negative controls, which did not contain DMO. As expected, a novel peak was present at the respective m/z (205) for DCSA in reactions containing both dicamba and DMO (Figure E-2C), confirming that the *E. coli*-produced MON 87429 DMO used for this analysis was functionally active. The data demonstrate that *o*-anisic acid was not catabolized by *E. coli*-produced MON 87429 DMO.

E.7. Conclusions

The data reported herein, together with the previously reported data (MON 87708 USDA-AHPIS Petition #10-188-01p) demonstrate that DMO, including *E. coli*-produced MON 87429 DMO, is selective for its substrate, dicamba.

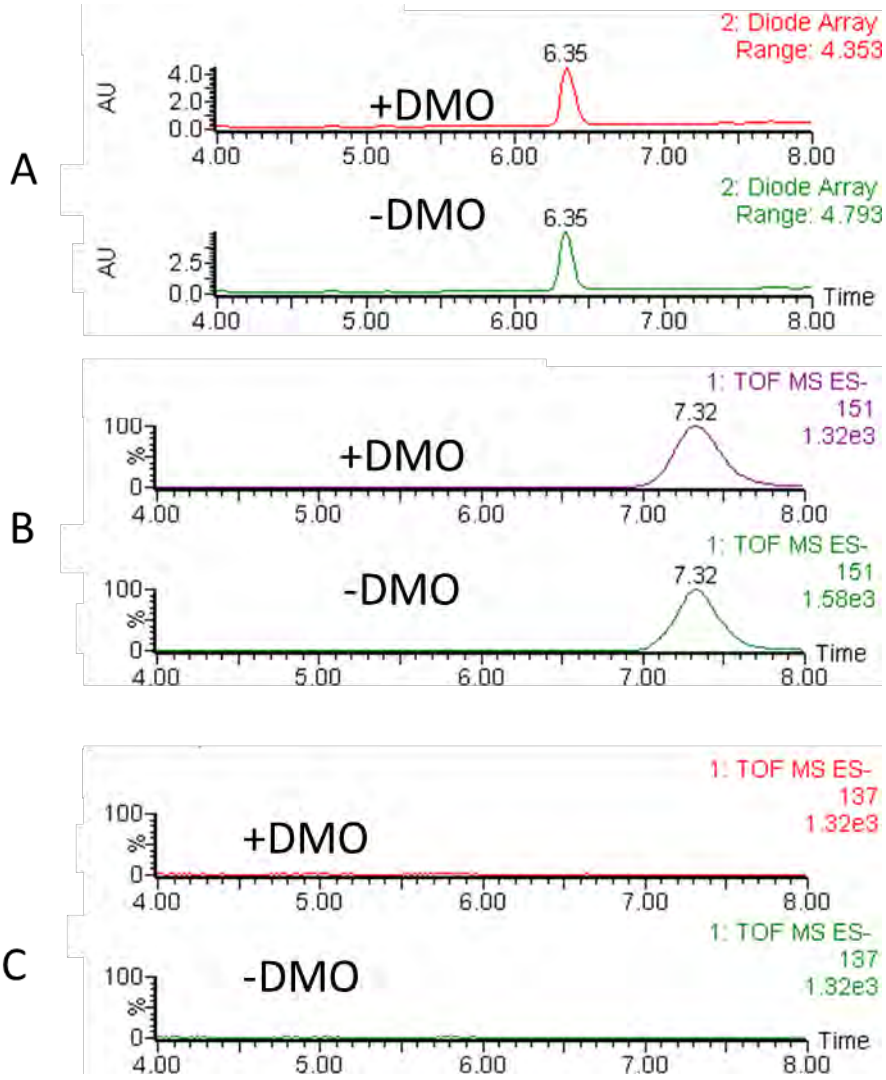


Figure E-1. Representative Assay Results for *o*-Anisic Acid

0.3 mM of *o*-anisic acid was included in a reaction mixture made with (+DMO, upper) or without (-DMO, lower) MON 87429 DMO, and the presence of the added compound and formation of the predicted demethylated product was monitored by LC-UV (Panel A) and LC-MS (Panels B and C). Panel B chromatograms with a 5 µl injection show the monitored m/z (151) for *o*-anisic acid, while the panel C chromatograms with a 5 µl injection show the monitored m/z (137) for the predicted product. Only peaks of interest that eluted between 4-8 min were shown in the figure.

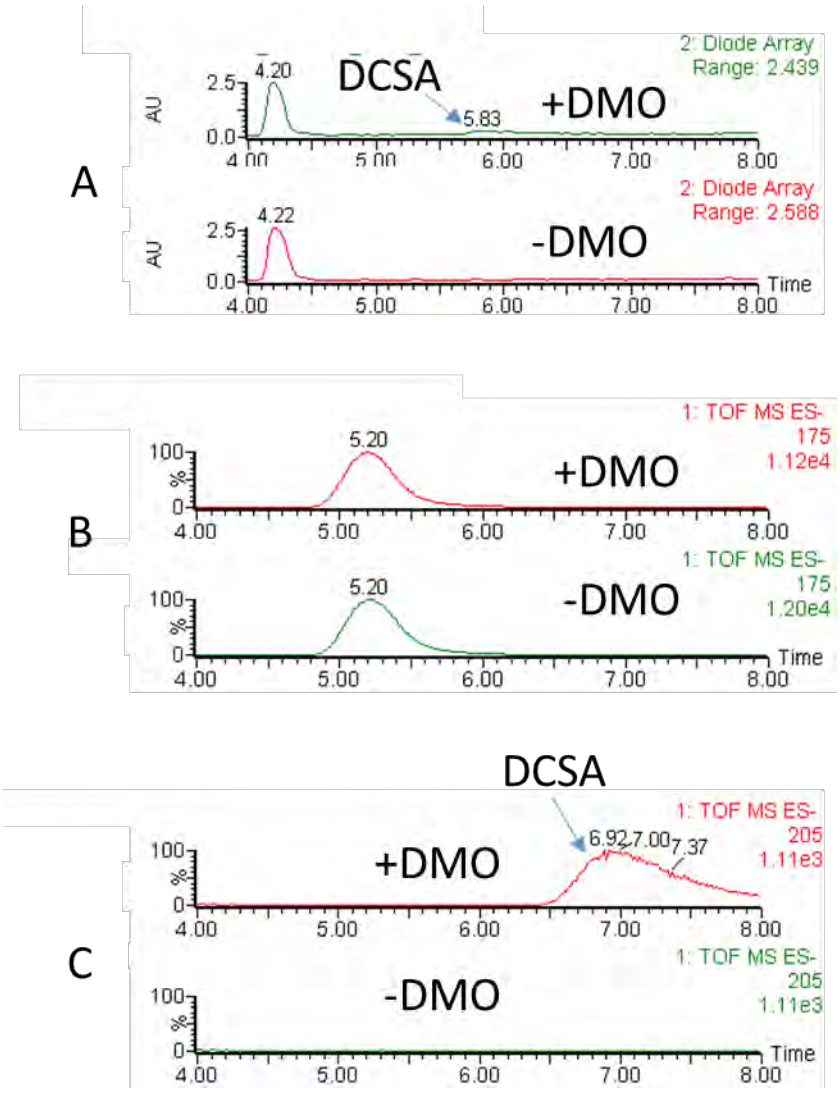


Figure E-2. Representative Assay Results for Dicamba

0.3 mM of dicamba was included in a reaction mixture made with (+DMO, upper) or without (-DMO, lower) MON 87429 DMO, and the presence of the added compound and formation of the expected demethylated product, DCSA, was monitored by LC-UV (Panel A) and LC-MS (Panels B and C). Panel B chromatograms with a 5 µl injection show the monitored m/z (175) for dicamba, while the panel C chromatograms with a 5 µl injection show the monitored m/z (205) for DCSA. The peak corresponding to DCSA is indicated by arrow in panels A and C. Only peaks of interest that eluted between 4-8 min were shown in the figure.

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Appendix F: Materials and Methods Used for the Analysis of the Levels of DMO, PAT, CP4 EPSPS and FT_T Proteins Expressed in MON 87429

F.1. Materials

MON 87429 forage, leaf, root, grain and pollen tissue samples were harvested from five sites grown in United States during the 2017 growing season from starting seed lot 11464934. CP4 EPSPS (10000739), PAT (11383593), FT_T (11462796) and DMO (11402310) were used as the analytical reference standards.

F.2. Characterization of the Materials

The identity of the test substance was confirmed by analysis of the starting seed DNA by an event-specific polymerase chain reaction method.

F.3. Field Design and Tissue Collection

The test substance was planted in four replicated plots at five sites using randomized complete block design.

F.4. Tissue Processing and Protein Extraction

The CP4 EPSPS, PAT, FT_T and DMO proteins were extracted from tissue samples as described in Table F-1.

Table F-1. DMO, PAT, FT_T and CP4 EPSPS Extraction Parameter

Sample Type[1]	Target Tissue to Buffer Ratio	Extraction Buffer
Forage, Leaf, Root, Grain and Pollen	1:100	TB pH 7.8[2]

[1] All proteins analyzed by Multiplexed Immunoassay were extracted by adding the appropriate volume of extraction buffer, stainless steel beads, and shaking in a Geno Grinder (SPEX Inc., Metuchen, NJ). The extracted samples were clarified by centrifugation.

[2] Tris borate buffer (pH 7.8) (0.1 M Tris, 0.1 M $Na_2B_4O_7$, 0.005 M $MgCl_2$, 0.05% (v/v) Tween 20)

F.5. Immunoassay Reagent and Methods

F.5.1. CP4 EPSPS Antibodies

Mouse monoclonal antibody clone 39B6.1 specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein A affinity chromatography. The concentration of the purified antibody was determined to be 5.9 mg/ml by spectrophotometric methods. The purified antibody (lot G-893017) was stored in a

phosphate buffered saline (1 × PBS, 15 mM NaN_3) and was used as the capture antibody in the multiplexed immunoassay.

Mouse monoclonal antibody clone 39B10.1.28 specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein A affinity chromatography. The purified antibodies were coupled with biotin (Thermo Fisher Scientific) per the manufacturer's instructions and assigned lot 209368-A. The detection reagent was streptavidin conjugated to R-Phycoerythrin.

F.5.2. PAT Antibodies

Purified mouse monoclonal antibody clone PAT (*pat*)-4 specific for PAT (*pat*) protein was purified by Protein G affinity chromatography. The concentration of the purified IgG was determined to be 0.9 mg/ml by spectrophotometric methods. The purified antibody (lot 276600E) was stored in 1 × PBS and was used as the capture antibody in the multiplexed immunoassay.

Purified rabbit polyclonal anti-PAT (*pat*) antibodies specific for the PAT (*pat*) protein were purified from serum using Protein G affinity chromatography and coupled with biotin per the manufacturer's instructions and assigned lot 351209. The detection reagent was streptavidin conjugated to R-Phycoerythrin.

F.5.3. FT_T Antibodies

Mouse monoclonal antibody clone 6-34.2.5 specific for the FT_T protein was purified by Protein G affinity chromatography. The concentration of the purified IgG was determined to be 2.95 mg/ml by spectrophotometric methods. The purified antibody (lot 546797) was stored in a phosphate buffered saline (1 × PBS, 15 mM NaN_3).

Mouse monoclonal antibody clone 6-35.2.11 specific for the FT_T protein was purified using Protein G affinity chromatography. The detection antibody was coupled with biotin (Thermo Fisher Scientific), per the manufacturer's instructions and assigned lot 546735.

F.5.4. DMO Antibodies

Mouse monoclonal antibody clone D712 specific for the DMO protein was purified from mouse ascites fluid using Protein A affinity chromatography. The concentration of the purified antibody was determined to be 3.3 mg/ml by spectrophotometric methods. The purified antibody (lot 151586) was stored in a phosphate buffered saline (1 × PBS, 15 mM NaN_3) and was used as the capture antibody in the multiplexed immunoassay.

Mouse monoclonal antibody clone 1-10C3.1.16.2 specific for the DMO protein was purified from mouse ascites fluid using Protein G affinity chromatography. The purified antibodies were coupled with biotin per the manufacturer's instructions and assigned lot 125874. The detection reagent was streptavidin conjugated to R-Phycoerythrin (Thermo Fisher Scientific).

F.6. Multiplexed Immunoassay Method

All CP4 EPSPS, PAT, FT_T and DMO proteins were analyzed using a validated multiplexed immunoassay method. Capture antibodies were covalently coupled to xMAP beads (Luminex Corp., Austin, TX) using the Antibody Coupling Kit (Luminex Corp., Austin, TX) as per the manufacturer's instructions. Antibody-coupled xMAP beads specific to each protein were diluted in sample buffer (1 × PBS containing 1% BSA (w/v)) to a final concentration of 50 beads/µl. Standards, QCs, and tissue samples were added to wells as appropriate followed by diluted beads and incubated for 30-60 minutes (min) at room temperature (RT) while shaking on a plate shaker at ~800. Plates were washed with 1 × PBS containing 0.05 % (v/v) Tween 20 (1 × PBST). A cocktail of biotinylated secondary antibodies was prepared in sample buffer containing 0.5% NFDM (w/v) and 0.5 mg/ml of mouse, rabbit, and goat IgG. The biotinylated antibody cocktail was added and incubated for 30-60 min at RT while shaking on a plate shaker at ~800. Plates were washed with 1 × PBST. Streptavidin RPE conjugate was added at a final concentration of 4 µg/ml and incubated for 30-60 min at RT by shaking on a plate shaker at ~800. Plates were washed with 1 × PBST. The beads were re-suspended by adding sample buffer and shaking on a plate shaker at ~800 for at least 10 min at RT. Quantification of each of the proteins was accomplished by interpolation from each of the protein standard curves.

F.7. Data Analyses

Multiplexed immunoassay plates for DMO, PAT, FT_T and CP4 EPSPS proteins were analyzed on the FLEXMAP 3D (Luminex Corp.). Plates were run as a batch on FLEXMAP 3D using appropriate methods, standards and control definitions. Data reduction analyses were performed using Milliplex Analyst software. Protein standard concentrations for each of the reference standards were fitted to a curve by the software using a best fit analysis (i.e. multiple models are fitted and the statistical best fit is used).

Following the interpolation from the standard curve, for data that determined to be greater than or equal to the LOQ, the protein levels (ng/ml) in the tissues were converted to a µg/g dw value. For each protein, this conversion utilized a sample dilution factor and a tissue-to-buffer ratio in Core Informatics Laboratory Information Management System (LIMS, version 5.1.28).

The across-site sample means, standard errors (SEs), and ranges were calculated by Microsoft Excel 2016.

One grain sample from IAAU site of MON 87429 resulted in an unexpectedly negative result for CP4 EPSPS protein. The sample was re-extracted twice and re-analyzed for CP4 EPSPS protein to confirm the results. The sample was found to be expressing slightly above LOQ and was included in calculations.

Appendix G: Mode of Action and Specificity of MON 87429 FT_T Protein

G.1. Materials and Methods Used for FT_T Enzyme Kinetics Characterization and Protein Melting Temperature Determination

Enzyme kinetics characterization was conducted using a modified phenolic-based colorimetric detection assay (Fukomori, F. et al. 1993). Purified proteins were tested with selected substrates. All substrates were obtained from Sigma Aldrich in the racemic form, except where indicated; all other reaction components were obtained from Sigma Aldrich. The reaction buffer contains 20 mM MOPS pH 6.75, 50 µM (NH4)2 $Fe(SO_4)_2$, 50 µM Ascorbic Acid (Na salt), and 1 mM alpha-ketoglutarate. Reactions were performed in 150 µl final reaction volumes in 96-well assay plates. All reactions for kinetic characterization were completed with four replications. Reactions were stopped with 15µl of stop solution (50 mM Boric Acid, 50 mM KCl, pH 10.0 (KOH) with 2% 4-aminoantipyrine w/v (in water) freshly diluted 1:10 into stop buffer). Color development agent was added (15 ul of an 8% potassium ferricyanide w/v (in water), freshly diluted 1:10). Following color development, assays were recorded with a SpectraMax spectrometer at an absorbance of 510 nm. Back calculations were completed with product standards in mock reactions (standards from Sigma Aldrich or custom synthesis). Control reactions without alpha-ketoglutarate were inactive, and reactions with purified enzyme without $(NH_4)_2$ $Fe(SO_4)_2$ and ascorbic Acid were significantly inhibited. Unless indicated, all reactions were completed at 23 °C.

Protein melting temperatures were determined by fluorescence tracking of the hydrophobic protein binding dye, SYPRO Orange (Invitrogen) with progressive increases in temperature. The assays were run in a 20 mM Tris, pH 7.75 and 150 mM NaCl buffer (with or without the addition of $(NH_4)_2$ $Fe(SO_4)_2$ and Ascorbic Acid, as in the enzyme reaction) containing the FT_T protein and a 1:100 dilution of SYPRO Orange dye in a Bio-Rad CFX96 PCR thermocycler with a fluorescence detector. The temperature was progressively increased from room temperature to full protein denaturation. The temperature at which the protein was 50% denatured was calculated.

Table G-1. Characterization of FT_T Enzyme

			RdpA	FT_T
	Protein melting	Buffer	43	58
		Buffer (Fe and αKG)	53	62
FOPs	Quizalofop	V_{max}	2.76 (0.11)	1.62 (0.05)
		K_m	0.09 (0.01)	0.12 (0.01)
	Haloxyfop	V_{max}	2.37 (0.05)	1.05 (0.02)
		K_m	0.06 (0.01)	0.04 (0.004)
Synthetic auxins	Dichlorprop	V_{max}	5.32 (0.09)	2.09 (0.03)
		K_m	0.06 (0.004)	0.03 (0.002)
	2,4-D	V_{max}	0.25 (0.01)	1.17 (0.01)
		K_m	0.13 (0.02)	0.03 (0.002)
	MCPA	V_{max}	0.20 (0.01)	1.23 (0.02)
	Mecoprop	V_{max}	4.11 (0.15)	3.04 (0.07)

FT_T and RdpA as a control are characterized for enzyme temperature stability and enzyme kinetics with FOP and synthetic auxin herbicides. Protein melting is recorded as the temperature (C) in which the protein is 50% denatured in buffer alone or with supplemental Fe_2 and α-ketoglutarate. Enzyme kinetic parameters are recorded for representative FOP and synthetic auxin herbicides with V_{max} (µmol mg^{-1} min^{-1}) and K_m (mM) shown. Standard error shown in parenthesis. ND, not determined.

G.2. Materials and Method for Endogenous Substrate Specificity Testing

The endogenous substrate screen utilized a computational screening method with chemical similarity and chemical docking screens followed by *in vitro* screening of the compounds that passed both computational screens and were thus the most likely candidates for substrates to the FT_T enzyme. The computational nature of the first two steps in this three-step screening process enabled screening of large datasets of small plant-based molecules in an efficient and robust manner. For these screens, a plant specific small molecule dataset from NAPRALERT (https://www.napralert.org/) (Bisson et al., 2016) was used (as of March 1, 2016). NAPRALERT is a relational database of natural products from different sources. Over 900 small molecules from maize, canola, cotton, wheat and soybean were identified from the literature and cataloged in this NAPRALERT dataset. Small molecules were curated digitally using publicly available tools (https://pubchem.ncbi.nlm.nih.gov/ and downloading the 2D sdf file).

A control database was also curated. The herbicide control database included 11 known substrates for the FT enzymes, including chemical ID: 15118048 (Cyhalofop), 7153 (Mecoprop), 7204 (MCPA), 86134 (Fenoxaprop), 91701 (Fluazifop), 1486 (2,4-dichlorophenoxyacetic acid), 41428 (Triclopyr), 50465 (Fluroxypyr), 50895 (Haloxyfop), 178795 (Quizalofop), 8427 (Dichlorprop). These compounds are not plant metabolites but are synthetic herbicide compounds.

For Step 1 of the screen (the compound similarity search) we internally solved the structure of FT_T bound to its substrate, dichlorprop. The details of the software parameters and coding are provided for computation methods below. The computational methods summarized here provide an overview of the process. The dichlorprop structure was used as the query with Blaze search engine from Cresset. The Blaze software searched our curated plant small molecule database for similar compounds based on electrostatic characteristics and shape (field print). The top 50% of the field print search from databases were refined using Clique and followed by simplex refinement using the top 50% from Clique refinement. The field similarity and 2D Tanimoto similarity were determined. From the output, only entries with field similarity greater than 0.6 and a Tanimoto coefficient greater than 0.1 were selected using Forge (no refinement was performed). These thresholds, which were chosen such that all herbicide control compounds were recovered, returned a total of 71 novel compounds from the original database.

Step 2 of the screens included docking and analysis of the selected compounds. To leverage the structural information of FT_T, we used protein-small molecule docking in Rosetta to evaluate the fitness of 71 candidate molecules within the active site of FT_T. The candidate molecules were superimposed onto the query (Dichlorprop) from Blaze. Conformations of the candidate molecule were generated from OpenBabel and Frog2. We used default methods from Rosetta Macromolecule Modeling Suite to generate molecular mechanics parameters and performed local docking and refinement of candidate molecules within the active site. A total of 1000 refinement decoys were generated using Rosetta ligand docking. Since the goal was to evaluate the fitness of

aligned candidate molecules within the protein active site after refinement, we selected the top 1% (10 decoys) with least centroid movement while maintaining favorable (negative) interaction energy between protein and ligand. We then analyzed several interface features between FT_T and the selected small molecules, including repulsion energy between protein and small molecule, coulombic interaction energy, and buried SASA (solvent accessible surface area). Using the averages of these three features, we selected cutoff values to recover all herbicide compounds. This process resulted 38 novel compounds that were as compatible as the putative substrate, and were selected as candidates for *in vitro* enzymatic screening.

All 38 compounds from the computational screening plus 11 control herbicide compounds and cinnamate (a compound identified as a marginal substrate in a similar enzyme family (Griffin, S. L. et al, 2013) were ordered through www.chemnavigator.com or Sigma Aldrich. Six of the compounds selected from Step 2 of the computational screens could not be screened *in vitro* because they were not commercially available or available from custom synthesis groups. Therefore, the in vitro enzymatic screening was conducted with a total of 44 compounds (11 herbicides and 32 compounds that passed the in-silico screen and cinnamate) (Table V-1).

For the final *in vitro* enzymatic screening of oxidation by FT_T of the selected compounds, we used a coupled enzyme screen because it uses a common detection system that does not require standards of the final reaction products. This assay was adapted from a coupled assay previously described (Luo et al. 2006). The reaction mixture contained 100 mM MOPS (pH 7.0), 1 mM PEP, 0.4 mM NADH, 0.4mM coenzyme A, 0.4 mM ATP, 0.1 mM $MgCl_2$, 0.1 mM $(NH_4)_2Fe\ (SO_4)_2$, 0.2 mM Na ascorbate, 1 µM succinate CoA synthetase, 6-10 units pyruvate kinase, 6-10 units lactate dehydrogenase, 2 µg recombinant purified FT_T and 200 µM of each test compound diluted from a 5 mM DMSO. All reagents were purchased from Sigma Aldrich except succinyl CoA Synthetase, which was purchased from Megazyme. Reactions were arrayed in a 96-well plate and pre-incubated at room temperature for 10 min. The reaction was started by the addition of 1 mM α-ketoglutarate. For measurement of both herbicides and putative endogenous substrates, the assay was performed in triplicate (each with 3 technical replicates for a total n=9, with fenoxaprop assayed twice for a total n=18) by measuring absorbance at 340 nm continuously for up to one hour using a TECAN spectrometer. A positive (2,4-D) and negative (blank; DMSO without a test compound) control were included on each plate.

To screen for false positive, all positive reactions were confirmed with control reactions using either no α-ketoglutarate or no FT_T enzyme reactions. In addition, all the compounds were monitored for solubility and absorbance spectra before testing to ensure that did not interfere with the assay. To ensure that any change in A_{340} was due to the oxidation of NADH rather than the direct oxidation of the compounds, all the compounds were tested in the assay without the coupling system. To ensure that the compound did not inhibit the coupling system producing a false negative effect, 1 mM succinate was added to each well that had a negative result and the A_{340} was compared to a control without additional succinate.

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Appendix H: Materials and Methods for Seed Germination and Dormancy Assessment of MON 87429

H.1. Materials

Seed germination and dormancy characteristics were assessed on seed from MON 87429 and the conventional control harvested from one 2017 field production site in Kihei, Hawaii, and on four reference maize hybrids obtained from commercial sources (Table H-1)

H.2. Characterization of the Materials

The presence of the MON 87429 event in the starting seed of the test material and the absence of the MON 87429 event in the starting seed of the conventional control were verified by event-specific polymerase chain reaction (PCR) analyses.

H.3. Germination Testing Facility and Experimental Methods

Germination and dormancy evaluations were conducted at SGS North America, Inc, Brookings, SD. The Principal Investigator was qualified to conduct seed germination and dormancy testing consistent with the standards established by the Association of Official Seed Analysts (AOSA, 2017a; b; AOSA, 2009; AOSA/SCST, 2010).

The seed lots of MON 87429, the conventional control, and four reference hybrids were tested under two temperature regimes. The optimum temperature regime consisted of alternating temperatures of approximately 20°C for 16 hours followed by 30°C for 8 hours and was maintained for seven days (AOSA, 2017a; b). The suboptimum temperature regime consisted of constant temperatures of approximately 10°C for seven days followed by 25°C for four days (AOSA, 2009). One germination chamber was used for each temperature regime. Each chamber was maintained dark. The temperature inside each germination chamber was monitored and recorded throughout the duration of the study.

Rolled germination towels were assembled for each material by placing approximately 50 seeds on a pre-moistened and labeled germination towel. An additional pre-moistened germination towel was placed on top of the seed, and the towels and seed were rolled up. Approximately 100 seeds (a set of two germination towel rolls secured together with a rubber band) of each material were placed into a bucket to form a replication. Each temperature regime constituted a separate experiment that was conducted using a randomized complete block design with four replications (*i.e.*, buckets).

Each set of rolled germination towels in each temperature regime was assessed at prescribed timings for germinated, dead, firm swollen (viable and nonviable), and hard (viable and nonviable) seed. Additional details for each germination characteristic evaluated and the timing of evaluations are presented in Table VII-1. Seeds placed under the optimum temperature regime were evaluated according to AOSA standards for testing

of maize (AOSA, 2017a; b). Seeds placed under the suboptimum temperature regime were evaluated according to AOSA standards for cold testing of maize (AOSA, 2009).

Within both the optimum and suboptimum temperature regimes, firm-swollen and hard seeds remaining at the final evaluation date were subjected to a tetrazolium (Tz) test for viability according to AOSA standards (AOSA/SCST, 2010). The numbers of non-viable firm-swollen and non-viable hard seeds were added to the number of dead seed counted on all collection dates to determine the total number of dead seed. Total numbers of viable firm-swollen and viable hard seed were determined from the Tz test.

The percentage of seed in each assessment category was based on the number of seeds evaluated. Across temperature regimes, the total number of seeds evaluated from each set of rolled germination towels was approximately 100.

H.4. Statistical Analysis

For each temperature regime, ANOVA was conducted according to a randomized complete block design using SAS® (SAS, 2012) to compare MON 87429 to the conventional control for the germination and dormancy characteristics. If analysis of variance assumptions were not satisfied, Fisher's Exact test was conducted for that characteristic using SAS (SAS, 2012). The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$). The reference range for each characteristic was determined from the minimum and maximum mean values among the four references.

Table H-1. Starting Seed for Germination and Dormancy Assessment of MON 87429

Material Name	Regulatory Lot Number	Phenotype	Material Type
MON 87429	11478364	Herbicide Tolerant	Test
LH244+HCL617	11478363	Conventional	Control
Dekalb DKC64-85	11464651	Conventional	Reference
Dekalb DKC 61-52	11427255	Conventional	Reference
Kruger K-0708	11427267	Conventional	Reference
Agrigold A 6300	11446938	Conventional	Reference

References for Appendix H

AOSA. 2009. Seedling evaluation. Rules for Testing Seeds. Volume 4. Association of Official Seed Analysts, Ithaca, New York.

AOSA. 2017a. AOSA Rules for testing seeds. Volume 1: Principles and procedures. Association of Official Seed Analysts, Washington, D.C.

AOSA. 2017b. AOSA Rules for testing seeds. Volume 4: Seedling evaluation. Association of Official Seed Analysts, Washington, D.C.

AOSA/SCST. 2010. Tetrazolium testing handbook. Association of Official Seed Analysts and the Society of Commercial Seed Technologists, Ithaca, New York.

SAS. 2012. Software Release 9.4 (TS1M4). Copyright 2002-2012 by SAS Institute, Inc., Cary, North Carolina.

Appendix I: Material and Methods for Phenotypic, Agronomic, and Environmental Interaction Assessment of MON 87429 under Field Conditions

I.1. Materials

Agronomic, phenotypic, and environmental interaction characteristics were assessed for MON 87429, the conventional control, and reference hybrids grown under similar agronomic conditions. Four reference hybrids were planted per site (Table I-1). A total of 16 unique reference hybrids were evaluated among the eight sites.

I.2. Characterization of the Materials

The presence of the MON 87429 event in the starting seed of the test material and the absence of the MON 87429 event in the starting seed of the conventional control were verified by event-specific polymerase chain reaction (PCR) analyses.

I.3. Field Sites and Plot Design

Field trials in 2017 at eight sites provided a range of environmental and agronomic conditions representative of U.S. maize growing regions. The Principal Investigator at each site was familiar with crop growth and production and evaluation of crop characteristics. The study was established at each site in a randomized complete block design with four replications. Plot and row dimensions are listed in Table I-2.

I.4. Planting and Field Operations

Planting information, soil description, and cropping history of the sites are listed in Table I-2. The Principal Investigator at each site followed local agronomic practices including those related to seed bed preparation and trial maintenance such as application of agricultural chemicals, fertilizer, and irrigation. All maintenance operations were performed uniformly across all plots within each site.

Table I-1. Starting Seed for Phenotypic, Agronomic, and Environmental Interactions Assessment of MON 87429

Site Code[1]	Material Name	Regulatory Lot Number	Phenotype	Material Type
All	MON 87429	11464934	Herbicide Tolerant	Test
All	LH244+HCL617	11464933	Conventional	Control
IAAU	Dekalb DKC61-52	11427255	Conventional	Reference
IAAU	Lewis 1407	11427264	Conventional	Reference
IAAU	Golden Harvest G12J11-A	11446928	Conventional	Reference
IAAU	LG Seeds LG2549	11446934	Conventional	Reference
IARL	Lewis 1613	11427265	Conventional	Reference
IARL	Stone 5820	11427266	Conventional	Reference
IARL	Agrigold A6472	11446939	Conventional	Reference
IARL	Agrigold A6574	11446940	Conventional	Reference
ILCX	Dekalb DKC64-85	11427257	Conventional	Reference
ILCX	Lewis 1613	11427265	Conventional	Reference
ILCX	Kruger K-0708	11427267	Conventional	Reference
ILCX	Mycogen Seeds MY09V40	11446930	Conventional	Reference
ILMN	Dekalb DKC61-52	11427255	Conventional	Reference
ILMN	Dekalb DKC65-18	11427258	Conventional	Reference
ILMN	Golden Harvest G09C43	11446927	Conventional	Reference
ILMN	Agrigold A6574	11446940	Conventional	Reference
INSH	Kruger K-0708	11427267	Conventional	Reference
INSH	Mycogen Seeds 2H721	11446932	Conventional	Reference
INSH	LG Seeds LG2549	11446934	Conventional	Reference
INSH	LG Seeds LG2636	11446935	Conventional	Reference
NESW	Lewis 1407	11427264	Conventional	Reference
NESW	Lewis 1613	11427265	Conventional	Reference
NESW	Golden Harvest G12J11-A	11446928	Conventional	Reference
NESW	Mycogen Seeds MY09V40	11446930	Conventional	Reference
NEYO	Dekalb DKC64-85	11427257	Conventional	Reference
NEYO	Golden Harvest G12J11-A	11446928	Conventional	Reference
NEYO	LG Seeds LG2549	11446934	Conventional	Reference
NEYO	Agrigold A6574	11446940	Conventional	Reference
OHTR	Dekalb DKC62-06	11427256	Conventional	Reference
OHTR	Dekalb DKC64-85	11427257	Conventional	Reference
OHTR	Kruger K-0708	11427267	Conventional	Reference
OHTR	Golden Harvest G09C43	11446927	Conventional	Reference

Note: Starting seed of test and control materials were produced in Kihei, HI in 2017.

[1] Site codes: IAAU = Audubon County, IA; IARL = Jefferson County, IA; ILCX = Vermilion County, IL; ILMN = Warren County, IL; INSH = Boone County, IN; NESW = Seward County, NE; NEYO = York County, NE; OHTR = Miami County, OH.

Table I-2. Field Information for Phenotypic, Agronomic, and Environmental Interactions Assessment of MON 87429

Site Code[1]	Planting Date[2]	Harvest Date[2]	Planting Rate (seeds/m^2)	Rows/ Plot	Inter-row Distance (cm)	Row Length (m)	Plot Size (m^2)	Soil Texture	Organic Matter (%)	Previous Crop 2016
IAAU	05/13/2017	11/01/2017	9.5	8	76	6.1	37.2	Silty Clay Loam	2.8	Soybean
IARL	05/05/2017	10/05/2017	9.5	8	76	6.1	37.2	Silty Clay Loam	3.4	Soybean
ILCX	06/01/2017	10/10/2017	8.6	8	76	6.2	38.1	Clay Loam	4.0	Maize
ILMN	05/09/2017	10/09/2017	9.5	8	76	6.1	37.2	Clay Loam	5.1	Soybean
INSH	05/18/2017	10/31/2017	9.5	8	76	6.1	37.2	Loam	2.2	Maize
NESW	05/08/2017	10/12/2017	9.5	8	76	6.1	37.2	Loam	3.1	Maize
NEYO	05/08/2017	10/19/2017	9.5	8	76	6.1	37.2	Loam	3.1	Soybean
OHTR	06/07/2017	11/17/2017	9.5	8	76	6.4	39.0	Loam	1.8	Maize

Note: Planting rate and plot/row dimensions are approximate.

[1] Site codes: IAAU = Audubon County, IA; IARL = Jefferson County, IA; ILCX = Vermilion County, IL; ILMN = Warren County, IL; INSH = Boone County, IN; NESW = Seward County, NE; NEYO = York County, NE; OHTR = Miami County, OH.

[2] Date format = mm/dd/yyyy.

I.5. Phenotypic Characteristic Assessments

Phenotypic characteristics assessed and the timing of each assessment are listed in Table VII-1.

I.6. Environmental Interaction Assessments

The test, conventional control, and commercial references were evaluated at each site for differences in plant responses to abiotic stressors, diseases, and arthropod pests. Evaluations were performed four times during the growing season at the following growth stages: V5 – V8, V12 – R1, R1 – R3, and R4 – R5.

The Field Co-operator at each site identified abiotic stressors, diseases, and arthropod pests that were either actively causing plant injury in the plots or likely to occur in maize during a given observation period. Stressors assessed often varied among observations and sites.

Ratings were made using the categorical scale of increasing severity listed below:

Category	Severity of plant damage
None	No symptoms observed
Slight	Symptoms not damaging to plant development (*e.g.,* minor feeding or minor lesions); mitigation likely not required
Moderate	Intermediate between slight and severe; likely requires mitigation
Severe	Symptoms damaging to plant development (*e.g.,* stunting or death); mitigation unlikely to be effective

I.7. Statistical Analysis/Data Summarization

A combined-site analysis was conducted according to a randomized complete block design using SAS® (SAS, 2012) to compare the test to the conventional control for the phenotypic characteristics listed in Table VII-1. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$). Descriptive statistics are provided for one characteristic, seed loss, that had insufficient variability for formal statistical analysis. The reference range for each phenotypic characteristic was determined from the minimum and maximum mean values among the 16 references, where each mean was combined over all the sites at which the reference was planted. There were no plots for which data were excluded from the study.

The environmental interactions data consisting of plant responses to abiotic stressors, diseases, and arthropod pests are categorical and were considered different in susceptibility or tolerance if the range of injury symptoms did not overlap between the

biotechnology-derived crop and the conventional control across all four replications within an observation.

I.8. Detailed Results for Environmental Interactions Assessments for MON 87429

No differences were observed between the test and the conventional control for any of the 96 comparisons of plant responses to the assessed abiotic stressors: cold temperatures, drought, excessive rain (i.e. waterlogging), hail, high temperatures, high winds (lodged plants), nutrient deficiency, soil compaction, and sun scald (Table I-3).

No differences were observed between the test and the conventional control for any of the 96 comparisons of damage from the assessed diseases: anthracnose, corn stunt, eyespot, Goss's bacterial wilt, gray leaf spot, leaf blight, northern leaf spot, rust, smut (head and ear), and Stewart's wilt (Table I-4).

No differences were observed between the test and the conventional control for any of the 96 comparisons of damage from the assessed arthropods: aphids, armyworms, billbugs, corn earworms, corn flea beetles, corn rootworm beetles, cutworms, European corn borers, grasshoppers, Japanese beetles, sap beetles, slugs, spider mites, and stink bugs (Table I-5).

The results of the assessed environmental interactions support the conclusion that MON 87429 is not expected to pose increased plant pest risk compared to conventional maize.

Table I-3. Summary of Abiotic Stressor Response Evaluations for MON 87429 and the Conventional Control in 2017 U.S. Field Trials

Abiotic Stressor	Total Observations across Sites	Number of Observations without Differences between the Test and the Conventional Control
Total	96	96
Cold Temperatures	1	1
Drought	13	13
Excessive Rain (i.e. waterlogging)	14	14
Hail	10	10
High Temperatures	10	10
High Winds (lodged plants)	26	26
Nutrient deficiency	12	12
Soil compaction	3	3
Sun scald	7	7

Notes: No differences were observed between the test and the conventional control during any observation for responses to any of the assessed abiotic stressors. Categorical data were summarized across sites and observation times.

Table I-4. Summary of Disease Damage Evaluations for MON 87429 and the Conventional Control in 2017 U.S. Field Trials

Disease	Total Observations across Sites	Number of Observations without Differences between the Test and the Conventional Control
Total	96	96
Anthracnose	8	8
Corn stunt	4	4
Eyespot	8	8
Goss's bacterial wilt	12	12
Gray leaf spot	20	20
Leaf blight[1]	13	13
Northern leaf spot	3	3
Rust[2]	18	18
Smut (head and ear)	8	8
Stewart's wilt	2	2

Notes: No differences were observed between the test and the conventional control during any observation for damage caused by any of the assessed diseases. Categorical data were summarized across sites and observation times.
[1] Includes Northern.
[2] Includes common.

Table I-5. Summary of Arthropod Damage Evaluations for MON 87429 and the Conventional Control in 2017 U.S. Field Trials

Arthropod	Total Observations Across Sites	Number of Observations without Differences between the Test and the Conventional Control
Total	96	96
Aphids (Aphididae)	12	12
Armyworms (Noctuidae)	13	13
Billbugs (weevil)	1	1
Corn earworms (*Helicoverpa zea*)	7	7
Corn flea beetles (*Chaetocnema pulicaria*)	7	7
Corn rootworm beetles (*Diabrotica* spp.)	12	12
Cutworms (Noctuidae)[1]	7	7
European corn borers (*Ostrinia nubilalis*)	2	2
Grasshoppers (*Melanoplus* spp.)	15	15
Japanese beetles (*Popillia japonica*)	10	10
Sap beetles (Nitidulidae)	1	1
Slugs[2]	1	1
Spider mites (*Tetranychus* spp.)	2	2
Stink bugs (Pentatomidae)	6	6

Notes: No differences were observed between the test and the conventional control during any observation for damage caused by any of the assessed arthropods. Categorical data were summarized across sites and observation times.

[1] Includes Western bean cutworm.

[2] Slugs are not arthropods but are occasional pests in maize.

References for Appendix I

SAS. 2012. Software Release 9.4 (TS1M4). Copyright 2002-2012 by SAS Institute, Inc., Cary, North Carolina.

Appendix J: Materials and Methods for Pollen Morphology and Viability Assessment

J.1. Plant Production

Pollen Morphology and Viability were assessed for MON 87429, the conventional control, and four conventional reference hybrids grown under similar agronomic conditions in a field trial in Warren County, Illinois in 2017 (Table J-1). The trial was arranged in a randomized complete block design with four replications. Each plot consisted of 8 rows approximately 6 m in length.

J.2. Pollen Collection and Pollen Sample Preparation

Pollen was collected from the three plants (tassels) per plot. The pollen collected from an individual tassel represented a subsample. Each subsample was transferred to a uniquely labeled tube containing approximately 400 µl of Alexander's stain solution (Alexander, 1980) diluted 1:5 with distilled water. The tubes were closed and shaken until thoroughly mixed. Pollen was allowed to stain and fix for at least 20 hours at ambient temperatures before assessments.

J.3. Data Collection

Slides were prepared by aliquoting suspended pollen/stain solution onto a slide. Pollen characteristics were assessed under an Olympus[Ó] BX53 light microscope equipped with an Olympus[©] DP72 digital color camera at a target magnification of 200x. The microscope and camera were connected to a computer running Microsoft Windows XP[Ò] and installed with Olympus© cellSens version 1.4.1 software.

J.3.1. Pollen Viability

When pollen grains are exposed to the staining solution, viable pollen grains stain red to purple due to the presence of living cytoplasmic content. Non-viable pollen grains stain light blue to green or colorless, and the shape can appear round to collapsed depending on the degree of hydration. For each pollen sample, the number of viable and non-viable pollen grains were counted from a random field of view under the microscope. A minimum of 125 pollen grains were counted for each of the three subsamples per plot. Mean pollen viability for each plot was calculated from the subsamples and expressed as a percentage of total number of evaluated pollen grains.

[©] Olympus Corporation.

[®] Windows XP is a registered trademark of Microsoft Corporation.

J.3.2. Pollen Diameter

For a single predetermined subsample from each plot, pollen grain diameter was measured along two perpendicular axes for 10 representative pollen grains. Mean pollen diameter for each plot was calculated from the total of 20 diameter measurements.

J.3.3. General Pollen Morphology

General pollen morphology was observed from micrographs of the test, control, and the reference materials that were also used for the pollen diameter measurements.

J.3.4. Statistical Analysis

An analysis was conducted according to a randomized complete block design using SAS® (SAS, 2012) to compare the test to the conventional control for percent viable pollen and pollen grain diameter. The level of statistical significance was predetermined to be 5% (α=0.05). The reference range for each phenotypic characteristic was determined from the minimum and maximum mean values among the four references. General pollen morphology was qualitative; therefore, no statistical analysis was conducted on these observations. There were no plots for which data were excluded from the study.

J.3.5. General Pollen Morphology Results

No visual differences in general pollen morphology were observed between MON 87429 and the conventional control (Figure J-1).

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Table J-1. Starting Seed for Pollen Morphology and Viability Assessment

Material Name	Material Type	Phenotype	Regulatory Lot Number
MON 87429	Test	Herbicide Tolerant	11464934
LH244+HCL617	Control	Conventional	11464933
Agrigold A6574	Reference	Conventional	11446940
Dekalb DKC61-52	Reference	Conventional	11427255
Dekalb DKC65-18	Reference	Conventional	11427258
Golden Harvest G09C43	Reference	Conventional	11446927

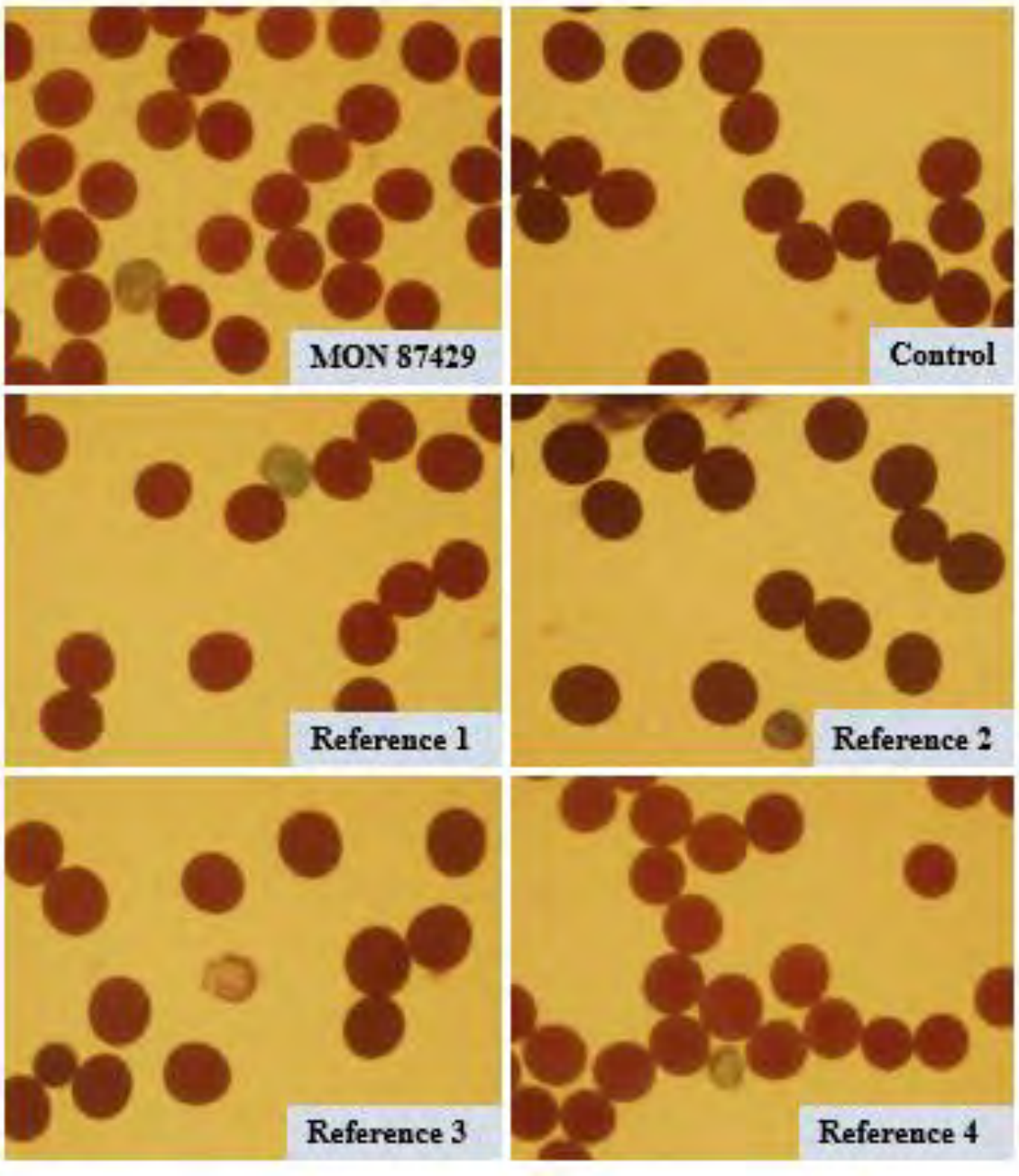


Figure J-1. General Morphology of Pollen from MON 87429, the Conventional Control, and Commercially Available Conventional Reference Materials under 200X Magnification

Viable pollen grains stained red to purple, while non-viable pollen grains stained lighter or colorless and the shape appeared round to collapsed depending on the degree of hydration.

References for Appendix J

Alexander, M.P. 1980. A versatile stain for pollen fungi, yeast and bacteria. Stain Technology 55:13-18.

SAS. 2012. SAS/STAT software version 9.3. SAS Institute, Inc., Cary, North Carolina.

Appendix K: Herbicide Resistance

K.1. Introduction

Based upon theory of natural selection, plant populations can develop resistance to an herbicide due to the selection of individuals that carry variations in genes that can render those individuals unaffected by the typical lethal effects of an herbicide. Over time, those few plant biotypes naturally containing resistant gene(s) become dominant in the population as they survive repeated use of the herbicide in the absence of other control methods (e.g., other herbicides with different mechanism of actions, mechanical, cultural or biological methods). The development of resistant populations is a possibility for all herbicides. The probability for resistance to develop is a function of: frequency of resistant allele(s)[11], mechanism of resistance, dominance or recessive nature of the resistant allele(s), relative fitness of the resistant biotype, and frequency or duration of herbicide use in the absence of other control methods (Beckie, 2006; Jasieniuk et al.,1996; Sammons et al., 2007). The probability of resistance is not the same for all herbicides, with some herbicides (e.g., ALS and ACCase classes) exhibiting resistance more quickly than other herbicides (e.g. auxin, glyphosate, glufosinate, and acetanilide, classes).

Herbicide resistance can become a limiting factor in crop production if the resistant weed population cannot be controlled with other herbicides, or with other methods of control. In general, this has not been the case for any herbicide. In most crops, there are multiple herbicide options for growers to use. However, good management practices to delay the development of herbicide resistance have been identified and are being actively promoted by the public and private sectors (HRAC, 2015; Norsworthy et al., 2012; WSSA, 2019)[12] and are being implemented by growers.

Monsanto considers product stewardship to be a fundamental component of customer service and business practices. Stewardship of dicamba, glufosinate, quizalofop, 2,4-D and glyphosate herbicides to preserve their usefulness for growers is an important aspect of Monsanto's stewardship commitment. Although herbicide resistance may eventually occur in weed species when any herbicide is widely used, resistance can be postponed, contained, and managed through implementation of good management practices by growers and associated research and education. These activities are key elements of Monsanto's approach to providing stewardship of dicamba, glufosinate, quizalofop,

[11] An allele is any of several forms of a gene, usually arising through mutations, that are responsible for hereditary variation.

[12] The Herbicide Resistance Action Committee (HRAC; www.hracglobal.com) is an international body founded by the agrochemical industry for the purpose of supporting a cooperative approach to the management of herbicide resistance and the establishment of a worldwide herbicide resistance database.

2,4-D and glyphosate used on MON 87429 maize that will be combined with deregulated full plant glyphosate tolerance traits, such as NK603. Monsanto will continue to invest in research, and grower/retailer education and training programs to provide information on best practices to delay the selection for weed resistance and to manage weeds already selected for resistance to dicamba, glufosinate, quizalofop, 2,4-D and glyphosate in maize production. This appendix provides an overview of Monsanto's approach to the development of best management practices to delay selection for weed resistance to dicamba, glufosinate, quizalofop, 2,4-D and/or glyphosate herbicides. Monsanto works closely with weed scientists in academia and with other companies to develop best management practices and to consistently communicate such practices to growers. An example of this collaboration is the development and posting of herbicide-resistance training modules on the WSSA website (www.wssa.net) and publication of guidelines by the Herbicide Resistance Action Committee (HRAC) on their website (www.hracglobal.com). The EPA is the U.S. federal regulatory agency that administers federal law governing pesticide sale and use under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). EPA recommends pesticide registrants to provide growers with information regarding an herbicide's sites-of-action and resistance management practices via labeling to aid growers in planning herbicide use practices and to foster the adoption of effective weed resistance management practices as specified by EPA in Pesticide Registration (PR) Notices 2017-1 and 2017-2 (U.S. EPA, 2017b; U.S. EPA, 2017c). Monsanto incorporates EPA's guidelines for pesticide site-of-action and resistance management labeling on its agricultural herbicide labels and will continue to do so in the future.

K.2. The Herbicides Dicamba and 2,4-D

Dicamba (3,6-dichloro-2-methoxybenzoic acid) is classified as a benzoic acid herbicide belonging to the synthetic auxin group of herbicides (WSSA, 2018). The herbicides in this group act as growth regulators similar to endogenous indole acetic acid (IAA) but are structurally diverse. The synthetic auxin group includes five chemical families (benzoic acid, pyridine-carboxylic acid, quinoline carboxylic acid, phenoxy-carboxylic acid and a separate class which includes one herbicide, benazolin ethyl). The specific site-of-action among the different synthetic auxin chemical families may be different. In addition to dicamba, other herbicides in the synthetic auxin group include 2,4-D, clopyralid, quinclorac and several other active ingredients. 2,4-D (2,4-Diclorophenoxy acetic acid) is classified as a phenoxy carboxylic acid herbicide (WSSA, 2018). As they regulate plant growth through an auxin receptor site-of-action, dicamba, 2,4-D and other synthetic auxin herbicides are classified in Herbicide Group 4 by the Weed Science Society of America (WSSA, 2018). Most herbicides in Group 4 are active on broadleaf weeds only, but a few have significant activity on grasses, e.g., quinclorac. Dicamba provides control of over 95 annual and biennial broadleaf weed species, approximately 50 perennial broadleaf species and control or suppression of over 50 woody species (Bayer CropScience, 2018). 2,4-D provides control of approximately 70 annual broadleaf and approximately 30 perennial broadleaf weed species (Dow, 2017). Dicamba and 2,4-D are not active on grass weeds and are often used in combination with other herbicides to provide broad spectrum weed control.

Dicamba was commercialized in the U.S. for agricultural use in 1967 and 2,4-D has been used as an herbicide since mid-1940s. They are currently labeled for use preplant and/or preemergence and/or postemergence to control emerged weeds in maize, sorghum, rice, small grains (wheat, barley, rye and oats), millet, pasture, rangeland, asparagus, sugarcane, turf, grass grown for seed, various other crops, conservation reserve program land, fallow cropland, and/or for non-crop uses (U.S. EPA, 2005; U.S.EPA, 2009). In addition, dicamba and 2,4-D are approved for preplant application in conventional soybean and cotton. Dicamba is approved for postemergence in-crop use in dicamba-tolerant soybean and cotton (Bayer CropScience, 2018), and 2,4-D is approved for postemergence in-crop use in 2,4-D-tolerant soybean, cotton and maize (Dow, 2017). Dicamba and 2,4-D are sold as standalone formulations which can be tank-mixed, based on the approved label, with one or more active ingredients depending upon the specific herbicide, the crop and the weed spectrum. Dicamba and 2,4-D are each sold as premix formulations with certain other herbicides.

K.3. The Herbicide Glufosinate

Glufosinate [2-amino-4-(hydroxymethylphosphinyl) butanoic acid] is classified by the Weed Science Society of America as a phosphinic acid herbicide belonging to the Group 10 glutamine synthetase inhibitor group of herbicides (WSSA, 2018). Bialaphos is the only other herbicide belonging to the phosphinic acid chemical family. Glufosinate provides control of approximately 70 annual broadleaf, 30 annual grass weed species and control or suppression of over 30 biennial and perennial grass and broadleaf weed species (Bayer CropScience, 2016).

Glufosinate was first registered for use in the U.S. in 1989 (U.S. EPA. 1989) and is currently labeled for non-crop uses, preplant burndown for glufosinate-tolerant and non-glufosinate-tolerant crops and/or in-crop postemergence weed control in glufosinate-tolerant canola, maize, cotton, and soybean (Bayer CropScience, 2016). Glufosinate is sold as a stand-alone formulation which can be tank mixed with other herbicides depending upon the specific herbicide, the crop and the weed spectrum.

K.4. The Herbicide Quizalofop

Chloroplastic acetyl-CoA carboxylase (ACCase) inhibitors Aryloxyphenoxypropionate (AOPP) and cyclohexanedione (CHD) herbicides have been widely used to control grass weed species since their introduction in the 1970s and 1980s, respectively (Kraehmer et al., 2014). These AOPP and CHD post-emergence grass herbicides are frequently referred to as “fops” and “dims”, respectively. While “fops” and “dims” herbicides are two chemically dissimilar classes of herbicides, they both inhibit the ACCase enzyme, which catalyzes the first committed step in fatty acid biosynthesis, causing plant death (Kraehmer et al 2014). Quizalofop-p-ethyl (Ethyl(*R*)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionic acid), is a systemic herbicide absorbed by the leaves with translocation throughout the plant (Mantzos et al., 2016). Quizalofop is classified as ACCase-inhibiting “fops” herbicide

belonging to the Herbicide Group 1 by the Weed Science Society of America (WSSA, 2018).

Quizalofop-p-ethyl was first approved for use as a registered product in 1990 (Assure® II: EPA Reg No. 352-541). It is used to selectively control post-emergent annual and perennial grass weeds in dicot crops such as potatoes, soybeans, peanuts, sugar beet, oilseed rape, sunflower, vegetables, cotton and flax and in quizalofop-tolerant maize (Wright et al., 2010; U.S. EPA, 2018).

K.5. The Herbicide Glyphosate

Glyphosate [N-(phosphonomethyl) glycine] is classified by the Weed Science Society of America as an EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) inhibitor herbicide belonging to the Group 9 (WSSA, 2018). Glyphosate is the only herbicide within Group 9 and is a non-selective, systemic, postemergence herbicide that translocates primarily to metabolic sinks, killing meristematic tissues away from the application site. Its phloem-mobile properties and slow action in killing weeds allow the herbicide to move throughout the plant, making it effective for weed control including perennial weeds (Duke 2018). Glyphosate provides control of approximately 100 annual weed species (grass and broadleaf), over 60 perennial weed species (grass and broadleaf) and control or suppression of approximately 65 woody brush, trees and vines (e.g., Roundup PowerMax® II; EPA Reg. No. 524-537).

Glyphosate was first registered for use in the U.S. in 1974 and has been extensively used globally (Duke, 2018). It is currently labeled for non-crop uses, preplant burndown to glyphosate- and non-glyphosate-tolerant crops and/or in-crop postemergence weed control in glyphosate-tolerant canola, maize, cotton, soybean, sugarbeet and alfalfa (Monsanto, 2017). Glyphosate is sold as a stand-alone formulation which can be tank mixed with other herbicides depending upon the specific herbicide, the crop and the weed spectrum. Glyphosate is also sold as premix formulations with certain other herbicides.

K.6. Herbicide-Resistant Weeds and Resistance Management Strategies

The development of herbicide-resistant weeds is not a new phenomenon and resistance is not limited to certain select herbicides. In 1957, the first U.S. herbicide-resistant weed, a spreading dayflower (*Commelina diffusa*) biotype resistant to 2,4-D, was identified in Hawaii (Heap, 2019). Through October 2018, there are approximately 160 unique cases of herbicide resistance by weed species with known herbicide-resistant biotypes to one or more herbicides in the U.S (Heap, 2019). Growers have been managing herbicide-resistant weeds for decades with the use of alternative herbicides and/or other methods of weed control. The occurrence of an herbicide-resistant weed biotype usually does not end the useful lifespan or preclude the effective use of the herbicide as part of an overall diversified weed management system but may change the way the herbicide is used in the cropping system.

As defined by the Weed Science Society of America, an herbicide resistant weed is one in which there is an inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type (WSSA, 2019). An herbicide-tolerant weed species is one that is naturally tolerant to an herbicide, for example a grass species is not killed by the application of a broadleaf herbicide (WSSA, 2019). Furthermore, certain weed species, while neither resistant nor tolerant, are inherently difficult to control with a particular herbicide, requiring tank mixing with other herbicides and/or other weed management practices.

Since the first confirmed cases of herbicide resistance, research has been directed at determining which practices are best for managing existing resistance situations and how best to reduce the development of further resistance or multiple herbicide resistance. Resistance management practices most often recommended by University/Cooperative Extension Service (CES) and industry are generally summarized as: a) Understand the biology of the weeds present; b) Use a diversified approach toward weed management focused on preventing weed seed production and reducing the number of weed seed in the soil seedbank; c) Plant into weed-free fields and then keep fields as weed free as possible; d) Plant weed-free crop seed; e) Scout fields routinely; f) Use multiple herbicide sites-of-action that are effective against the most troublesome weeds or those most prone to herbicide resistance; g) Apply the labeled herbicide rate at recommended weed sizes; h) Emphasize cultural practices that suppress weeds by using crop competitiveness; i) Use mechanical and biological management practices where appropriate; j) Prevent field-to-field and within-field movement of weed seed or vegetative propagules; k) Manage weed seed at harvest and after harvest to prevent a build-up of the weed seedbank; l) Prevent an influx of weeds into the field by managing field borders (Beckie, 2006; Gressel and Segel, 1990; Norsworthy et al., 2012; HRAC, 2015).

Recent research indicates that herbicide mixtures offer a better management option than rotating herbicides. However, the effectiveness of herbicide mixtures depends on their similar efficacies, soil residual activity and/or different propensities for resistance selection in the target weed species (Beckie and Reboud, 2009; Lagator et al., 2013; Evans et al., 2016; Beckie and Harker, 2017). Simultaneously using two herbicides with different sites-of-action, each effective on the same weed species, significantly reduce the probability of weeds developing resistance to either or both herbicides (Beckie and Reboud, 2009). Use of multiple methods of weed control including multiple herbicides with different sites-of-action in a single field is the technical basis for management programs to delay the development of resistance. This general concept has been referred to as applying “diversity” within a crop or across a crop rotation (Beckie, 2006; Powles, 2008; Andrew et al., 2015). While herbicide mixtures have been shown to be an effective strategy to delay the development of herbicide resistance, given that some weeds already evolved resistance to multiple previously effective herbicides, it could become challenging to find effective herbicide mixtures that individually suppress a specific weed species in the future (Evans et al., 2016). Broad use of herbicide mixtures may increase the chance of development of cross-resistance, especially if use rates are below the effective rates approved on the herbicide label (Evans et al., 2016). These challenges highlight the need to follow each best management practice described in the paragraph

above (a-l), including applying label rates of herbicides and using other cultural practices to assist in delaying the development of herbicide resistance.

K.7. Characteristics of Herbicides and Herbicide Use Influencing Resistance

While the incidence of weed resistance is often associated with repeated applications of an herbicide resulting in recurrent selection of the weed in the absence of other herbicides or methods of weed control, the actual probability for the development of resistant populations is related, in part, to the specific herbicide active ingredient, chemical family and herbicide group and especially the resistance mechanism. Some herbicides are more prone to the development of resistance than others (Heap, 2019; Figure K-1). The graph in Figure K-1 illustrates the global instances of weed resistance to various herbicide groups. The different slopes of observed resistance are largely due to the factors described above, which relate to the specific herbicide active ingredient as well as to the group and herbicide family and its function.

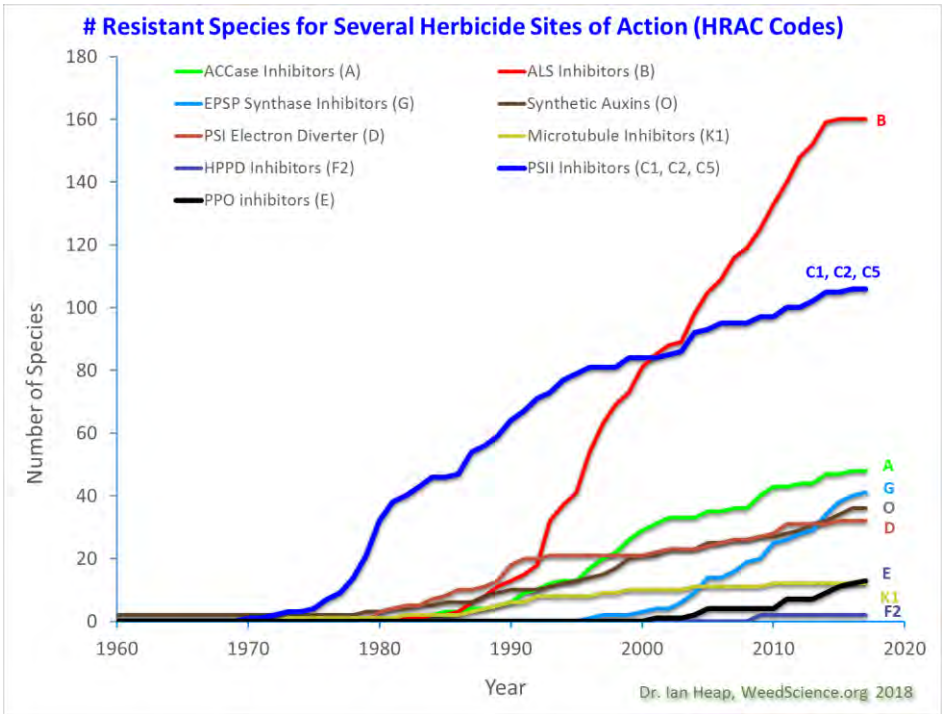


Figure K-1. Global Weed Resistance to Various Herbicide Families

Weed resistance to the synthetic auxins (O) group of herbicides has been slower to develop than for other herbicide groups even though these were the first synthetic herbicides discovered and used commercially. Possible reasons for this are discussed in this Section K-8.

K.8. Weeds Resistant to Dicamba, Glufosinate, Quizalofop, 2,4-D or Glyphosate Herbicides

As noted earlier in Section K.7, like other herbicides, the use of dicamba may lead to the development of dicamba-resistant weed species. To date, there are four species with known resistant biotypes to dicamba in the North America after over 40 years of use: common hempnettle, kochia, prickly lettuce and wild mustard (Heap, 2019). Additionally, a population of common lambsquarters has been confirmed to be resistant in New Zealand, wild mustard in Turkey, cornflower in Poland and smooth pigweed in Argentina have been confirmed to be resistant for a total of seven species worldwide with confirmed resistant biotypes to dicamba. For the synthetic auxin group of herbicides, there exist a total of appropriately 30 broadleaf species globally with biotypes having confirmed resistance to at least one member of this group, but only seven broadleaf species in the U.S. and six broadleaf species in Canada (Heap, 2019). Broadleaf weed species that are resistant to synthetic auxins in the U.S. include Palmer amaranth, tall waterhemp, yellow starthistle (*Centaurea solstitialis*), wild carrot, kochia, prickly lettuce and buckhorn plantain. In Canada, kochia, wild mustard, wild carrot, common hempnettle, spotted knapweed (*Centaurea maculosa*) and false cleavers (*Galium spurium*) were reported to be resistant to synthetic auxins. For 2,4-D herbicide, a total of 5 broadleaf weeds (Palmer amaranth, waterhemp, prickly lettuce, wild carrot, and buckhorn plantain) have been confirmed to be resistant in the U.S. All the broadleaf populations are found in the Midwest (Palmer amaranth in KS, waterhemp in NE and IL and wild carrot in OH and MI, and buckhorn plantain in IN), western U.S. (prickly lettuce in WA) or western Canadian provinces. In some weed species, cross-resistance between different herbicides within the auxin group has been confirmed (plant cross-resistance to another herbicide because of exposure to a similarly acting herbicide). Therefore, consideration must be given to the possibility that dicamba and/or 2,4-D resistance could extend to some of the other broadleaf species listed as resistant to other synthetic auxin herbicides (Cranston et al., 2001; Jasieniuk et al., 1995; Miller et al., 2001; Tehranchian et al., 2017). However, because of differences in sites-of-action among the chemistry families within the synthetic auxin herbicide group (i.e., benzoic acids compared to pyridine-carboxylic acids), cross-resistance between them is uncommon (Beckie and Tardif, 2012).

MON 87429 maize will be combined with deregulated glyphosate tolerance traits, such as NK603, where dicamba or 2,4-D can be applied in combination with glyphosate and glufosinate. It is important to note that in the U.S., kochia is the only broadleaf species with biotypes with herbicides resistance to dicamba, 2,4-D and glyphosate (LeClere et al., 2018; Heap, 2019). In addition, dicamba (only) resistant kochia biotypes were reported in 4 other states (Nebraska, Colorado, Idaho and North Dakota). In Kansas, 2 separate kochia biotypes, one with multiple resistance to dicamba, glyphosate, atrazine and chlorsulfuron and another with multiple resistance to dicamba, fluroxypyr and glyphosate were reported. A kochia biotype in Montana was reported to be resistant to dicamba and fluroxypyr (Heap, 2019). A biotype of waterhemp in Illinois was reported to be resistant to 2,4-D, HPPD, PS II, ALS and PPO inhibitors (Heap, 2019). In Nebraska, a waterhemp biotype was reported to be resistant to 2,4-D, aminopyralid, atrazine, chlorimuron,

imazethapyr and picloram (Heap, 2019). Biotypes of waterhemp with resistance to 2,4-D or glyphosate exist both in Illinois and Kansas (Heap 2019), and there is a high potential for biotypes with resistance to both herbicides to occur via crossing in this cross-pollinated dioecious species. A Palmer amaranth biotype in Kansas was reported to be resistant to both 2,4-D and glyphosate (also resistant to HPPD, PS II and ALS inhibitors) (Heap, 2019). In addition, a prickly lettuce biotype was reported to be resistant to 2,4-D and dicamba and MCPA in Washington state, outside of the main corn-growing regions of the U.S. (Heap, 2019). MON 87429 maize hybrids combined with deregulated glyphosate tolerance traits, such as NK603, provide tools to reduce the likelihood of resistance development in many broadleaf weed species and/or delay the likelihood of further selection or spread of biotypes that are already resistant to one or more herbicides with different sites-of-action. For example, growers would have options to rotate herbicides or use herbicide mixtures with dicamba or 2,4-D, glyphosate, and/or glufosinate, each with a distinct site-of-action, based on the approved label and the weed spectrum (Table VIII-7) to reduce the likelihood of resistance development. If additional weed populations with resistance to both glyphosate and dicamba or 2,4-D herbicides were to occur there are other preplant and/or postemergence herbicide options for managing the weed in maize and in its rotational crops (Table K-1).

There is no known broadleaf weed species reported to be resistant to glufosinate in the U.S (Heap, 2019). To date there are three grass weed species with confirmed resistance to glufosinate globally: goosegrass in Malaysia, rigid ryegrass in Greece, perennial ryegrass in New Zealand and Italian ryegrass in New Zealand and the U.S. (in Oregon and California states) (Heap, 2019). In the case of goosegrass (*Eleusine indica*), a population was found in Malaysia that is resistant to glufosinate and paraquat herbicides. Resistance to glufosinate evolved likely due to the repeated use of glufosinate for at least six times a year and for more than four consecutive years as standalone or in combination with paraquat in a bitter gourd field. It is speculated that paraquat resistance in this goosegrass population might have evolved earlier than glufosinate resistance since this vegetable field which was originally a rubber plantation had exposure to paraquat treatment since the 1970s (Seng et al., 2010). In rigid ryegrass (*Lolilum rigidum* Gaud.), glufosinate resistance was confirmed in Greece with the level of resistance ranging from three-to seven-fold. It was also demonstrated that the level of glufosinate-resistance of rigid ryegrass was dependent on the growth stage at which it was applied (Travlos et al., 2018). In the case of an observed Italian ryegrass biotype (*Lolium perenne ssp. multiflorum*), glufosinate resistance was discovered in populations not previously exposed to the herbicide. Avila-Garcia and Mallory-Smith (2011) found that resistance was not due to an insensitive or altered target site and hypothesized that reduced translocation is responsible for the resistance to both glyphosate and glufosinate in these populations. No resistance to glufosinate in a broadleaf species has been reported to date (Heap, 2019).

In the case of AOPP ACCase inhibitor “fops” (i.e. quizalofop) herbicides, johnsongrass, Italian ryegrass, giant foxtail, cheatgrass and common wild oat have been reported to be resistant in the U.S. (Heap, 2019). Certain biotypes of johnsongrass, Italian ryegrass, giant foxtail and cheatgrass were confirmed to be resistant to “fops” and/or “dims”

ACCase inhibitor herbicides. ACCase-resistant johnsongrass biotypes in the U.S. were reported in 5 states (Mississippi, Kentucky, Tennessee, Virginia and Louisiana), with 2 of these states (Mississippi and Louisiana) having different biotypes that are resistant to glyphosate (Heap, 2019). In addition, ACCase-resistant Italian ryegrass biotypes in the U.S. were reported in 13 states and biotypes in California were also resistant to glyphosate (Heap, 2019). However, these weeds are controlled by using other effective herbicides such as photosystem I and II inhibitors, HPPD (4-hydroxyphenylpyruvate dioxygenase) inhibitors, long-chain fatty acid inhibitors or glufosinate and/or glyphosate on non-glyphosate-tolerant grasses depending upon the crop and the weed spectrum. See Table K-1 for other herbicide options in maize and its rotational crops to control "fops"-resistant grass weeds.

For glyphosate, there are 44 weed species reported to be resistant globally (Heap, 2019). In the U.S. there were 17 weed species reported to be glyphosate resistant, six of those being grass species (junglerice, goosegrass, Italian ryegrass, rigid ryegrass, annual bluegrass and johnsongrass) and 11 broadleaf weed species (Palmer amaranth, waterhemp, common ragweed, giant ragweed, kochia, spiny amaranth, horseweed, hairy fleabane, common sunflower, ragweed parthenium and Russian thistle) (Heap, 2019). Many of the glyphosate-resistant broadleaf weeds, particularly, Palmer amaranth, horseweed, waterhemp, common ragweed, giant ragweed and kochia were found in at least 10 states in the U.S (Heap and Duke, 2018). Palmer amaranth was predominantly found in the southern U.S. states and glyphosate-resistant Palmer amaranth biotypes have been found in 24 states in the U.S. (Heap and Duke, 2018; Heap, 2019). Furthermore, waterhemp was more commonly seen in northern U.S. states (Heap and Duke, 2018) with biotypes resistant to glyphosate reported in 18 states (Heap, 2019).

K.9. Mechanisms of Resistance and Inheritance of Resistance

To date, three basic mechanisms by which weed species develop resistance to an herbicide have been identified: 1) target site alteration (point mutations and/or increased expression), 2) enhanced metabolism of the herbicides (via chemical modification of the herbicide either by conjugation or degradation), and 3) reduced herbicide access to the site-of-action within the plant cell (exclusion) (Sammons et al., 2007; Sammons and Gaines 2014). Avoidance was proposed as an additional mechanism of resistance whereby weeds avoid toxic effect by its biochemical ability to handle the toxic agent produced by the herbicide (Sammons and Gaines 2014).

Over 200 distinct weed biotypes worldwide have developed herbicide resistance with the most common resistance mechanism occuring via target site alteration (Devine and Shukla, 2000). It has been found that target site resistance is the most common mechanism for ALS inhibitors, ACCase inhibitors, and triazines, but is less common for other herbicide groups, such as glyphosate and synthetic auxins (Devine and Shukla, 2000; Sammons et al., 2007; Sammons and Gaines, 2014; Powles and Yu, 2010; Busi et al 2017). The most common type of target site alteration is one where amino acid substitution(s) occur in the protein that is the target of the herbicide such that the alteration prevents herbicide binding to the protein and as a result the activity of the

targeted protein is not altered, and the plant grows normally (Devine and Shukla, 2000). Recently five species (*Bassia scoparia*, *Amaranthus spinosus, Amaranthus palmeri*, *Amaranthus tuberculatus*, *Lolium perenne* ssp *multiflorum*) have managed to duplicate the EPSPS gene creating much higher expression levels of EPSPS protein (Sammons and Gaines, 2014; Koo et al., 2018a; Koo et al., 2018b).

In the case of synthetic auxin herbicides, there is limited evidence for any single mechanism conferring weed resistance and known evidence indicate different resistance mechanisms between weed species and/or biotypes within a weed species. Resistance to synthetic auxins has been speculated to be due to mutation(s) in genes encoding an auxin-binding protein, *ABP1* causing reduced herbicide binding or due to impaired auxin signal transduction pathway, including transport inhibitor response gene, *TIR1* (Zheng and Hall, 2001; Goss and Dyer, 2003; Grossmann, 2010). In several studies, differential herbicide absorption, translocation, and metabolism were ruled out as possible mechanisms of resistance to certain auxinic herbicides in kochia (Cranston et al., 2001) and in wild mustard (Zheng and Hall, 2001). In a recent study, it was demonstrated that a single amino acid change from glycine to asparagine in a conserved region within *KsIAA16* gene (a homologue of *IAA16* in arabidopsis) in a kochia biotype found in Nebraska, could result in cross-resistance between dicamba, 2,4-D and fluroxypyr herbicides (LeClere et al., 2018). This conserved protein domain in IAA16 was previously shown to be important for auxin binding and interaction between TIR1/F-box proteins and AUX/IAA in Arabidopsis (Tan et al., 2007). Pettinga et al., (2017) have shown that increased expression of *CHS* (*chalcone synthase*) gene in a resistant kochia biotype found in Nebraska (the same biotype studied by LeClere et al., 2018), co-segregated with dicamba resistance primarily by reducing dicamba translocation while no change was observed in herbicide uptake. The 2,4-D-resistant prickly lettuce biotype appears to have evolved resistance to other auxins in the phenoxy-carboxylate and benzoate subgroups, including MCPA and dicamba (Lyon and Burke 2016). Inheritance of 2,4-D resistance in prickly lettuce was governed by a single codominant gene and it was observed that the resistant biotype showed reduced translocation of 2,4-D in the treated leaf or crown but showed no difference in rate of 2,4-D metabolism compared to the susceptible biotypes (Riar et al 2011). Walsh et al., (2006) identified seven alleles at two distinct genetic loci that conferred significant resistance to picolinate auxins (picloram) in arabidopsis yet had minimal cross-resistance to 2,4-D and IAA, a naturally occurring plant growth regulator. Jasieniuk et al. (1995) reported results indicating that inheritance of dicamba resistance in wild mustard is determined by a single, completely dominant nuclear allele. While wild mustard biotype was highly resistant to dicamba, it was moderately resistant to 2,4-D indicating differences in resistance phenotypes for herbicides within the synthetic auxin site-of-action (Zheng and Hall, 2001). However, Cranston et al. (2001) reported results indicating that dicamba resistance in kochia biotype (found in Montana) is a quantitative trait (two or more genes). The slow development of weed resistance to synthetic auxin herbicides may in part be due to their proposed multiple sites of physiological action in plants (Jasieniuk et al., 1996) and to the possibility that inheritance, at least in some species, is a quantitative trait (Cranston et al., 2001). It is also demonstrated that reduced

fitness due to the auxin resistance in the kochia biotype found in Nebraska may be partially responsible for the rarity of auxin resistant weeds (LeClere et al., 2018).

Little is known about the resistance mechanisms in glufosinate-resistant biotypes. Avila-Garcia and Mallory-Smith (2011) conducted an initial set of experiments to understand the mechanism of resistance in the ryegrass population collected from Oregon that was also resistant to glyphosate. They found that resistance was not due to an insensitive or altered target site and hypothesized that reduced translocation is responsible for the resistance to both glyphosate and glufosinate in these populations. In another study conducted with an Italian ryegrass biotype resistant to glufosinate, it was shown that resistance to glufosinate was due to the target site mutation in plastidic *GS2* (*Glutamine Synthetase2*) gene causing a single amino acid change from asparagine to aspartic acid (Avila-Garcia et al., 2012). In a more recent study, Jalaludin et al. (2017) demonstrated that glufosinate resistance in resistant goosegrass population was not due to an insensitive glutamine synthase (GS), or increased GS activity, or altered glufosinate uptake and translocation, or enhanced glufosinate metabolism. They excluded the possibility of target-site resistance to glufosinate in goosegrass, however, the exact resistance mechanism(s) remain to be determined.

For ACCase inhibitors including “fops” and “dims” different patterns of resistance may be conferred by separate mutations in the gene for plastidic ACCase (Devine and Shulka 2000). A single modification (a single isoleucine-to-leucine substitution) confers cross-resistance to the “fops” and “dims” herbicides in wild oat (Christoffers et al., 2002), rigid ryegrass (Delye et al., 2002b; Tal and Rubin 2004; Zagnitko et al., 2001), green foxtail (Delye et al., 2002c), and blackgrass (*Alopecurus myosuroides* Huds.) (Delye et al., 2002a; Delye et al., 2002b). This isoleucine-to-leucine substitution at the 1781 position in *ACCase* gene in blackgrass confers resistance to certain “fops” (fenoxaprop and diclofop) and to “dims” (cycloxydim) and does not confer resistance to haloxyfop and clodinafop, nor to clethodim (Delye et al., 2002a; Delye et al., 2003b). A second substitution (an isoleucine-to-asparagine substitution) at position 2041 was associated with resistance to “fops” (fenoxaprop, diclofop, clodinafop and haloxyfop) but not “dims” (cycloxydim and clethodim) in blackgrass (Delye et al., 2003a). Both isoleucine-to-leucine substitution at the 1781 position and isoleucine-to-asparagine substitution at position 2041 in *ACCase* gene were dominant alleles (Delye et al., 2002a; Delye et al., 2003a) and arose independently in geographically distant blackgrass populations (Delye et al., 2003b). A recent study on large crabgrass (*Digitaria sanguinalis*) biotype collected from Ontario, Canada revealed that over expression of the ACCase enzyme conferred resistance to the ACCase inhibitor herbicides (both “fops” and “dims”) and that over expression of the ACCase enzyme in the resistant biotype was likely due to *ACCase* gene duplication (Laforest et al., 2017). Another large crabgrass biotype collected from South Australia that is resistant to “fops” (fluazifop, haloxyfop and quizalofop) and has reduced sensitivity to sethoxydim herbicides, was studied for mechanism of resistance to fluazifop. This study revealed that an enhanced metabolism of fluazifop acid conferred resistance to fluazifop herbicide rather than reduction in ACCase enzyme sensitivity, reduced absorption or translocation (Hidayat and Preston, 1997).

Studies of resistance to glyphosate have revealed a wider range of molecular mechanisms of resistance than are known for any other herbicide site-of-action (Sammons and Gaines, 2014), including herbicide sequestration in the vacuole, reduced translocation presumably due to a rapid cell death response, genetic polymorphisms in the herbicide target (5-enolpyruvylshikimate-3-phosphate synthase, EPSPS) and increased copy number for the gene encoding EPSPS. It was further observed that resistance mechanism due to copy number of *epsps,* had two distinct molecular mechanisms, a tandem duplication in kochia (Jugulam et al., 2014) and a large extrachromosomal circular DNA (eccDNA) that is tethered to the chromosomes and passed to gametes at meiosis in Palmer amaranth (Koo et al., 2018a). Target-site mutations in *epsps* have been documented at amino acid position Pro106 (to primarily Ser, Thr or Ala) in few weed species including *Poa annua* (Cross et al., 2015)*, Eleusine indica, Lolium rigidum, Lolium multiflorum, Echinochloa colona, Digitaria insularis, Amaranthus tuberculatus* (Sammons & Gaines, 2014), at Thr102Ser in *Tridax procumbens* (Li et al., 2018), at Thr102Ile and Pro106Ser in combination in *Eleusine indica* (Yu et al., 2015), and recently a triple mutation involving Thr102Ile, Ala103Val, and Pro106Ser in *Amaranthus hybridus* (Perotti et al., 2019). The simultaneous occurrence of multiple mutations within the same allele appears to be unique to glyphosate resistance and *epsps* (Gaines et al., 2019).

K.10. Sustainable Use of Dicamba, Glufosinate, Quizalofop, 2,4-D and Glyphosate as a Weed Management Option in Maize

Diversified use of herbicides with different and effective sites-of-action is one of the key weed management strategies to minimize selection for herbicide resistance. MON 87429 maize will likely be sold in maize hybrids that also contain other herbicide-tolerant traits, including glyphosate-tolerance. Maize hybrids containing both MON 87429 and glyphosate tolerance traits, such as NK603, will enable dicamba, glufosinate, quizalofop or 2,4-D herbicides to be applied with glyphosate and/or other maize herbicides depending upon the specific herbicide use labels and the weed spectrum in an integrated weed management program. Either dicamba or 2,4-D will likely be used in mixtures with glyphosate or in sequence with glyphosate or glufosinate to control a broad spectrum of grass and broadleaf weed species. Glyphosate and glufosinate will likely not be used in mixtures due to antagonism (i.e., glufosinate damages the leaf tissue before glyphosate gets into the plant and/or translocated to growing parts of the plant) and reduced efficacy of glyphosate on susceptible weed species. Either dicamba or 2,4-D and glufosinate applications on MON 87429 maize will provide effective control of glyphosate-resistant broadleaf weeds and improve the control of annual and perennial broadleaf weed species, some of which are difficult to control with glyphosate. Either dicamba or 2,4-D and glufosinate (Ganie and Jhala, 2017; Joseph et al., 2018) will also help delay development and/or combat existing weed resistance issues that can limit the use of other herbicides such as glyphosate. Likewise, dicamba or 2,4-D with or without glyphosate (depending on the presence of glyphosate- resistant weeds) may help delay or minimize potential evolution of resistance to glufosinate in broadleaf species and similarly, glufosinate may delay or minimize the potential evolution of broadleaf species biotypes resistant to dicamba, 2,4-D or glyphosate (Ganie and Jhala, 2017; Joseph et al., 2018). In addition, residual herbicides also will be recommended for use, to provide early season weed

control and to supplement dicamba or 2,4-D and glufosinate activity on certain hard-to-control and glyphosate-resistant weed biotypes, such as glyphosate-resistant Palmer amaranth where weed population density can be very substantial. However, over-reliance on any herbicide(s) for weed control including dicamba, 2,4-D, glufosinate, "fops" or glyphosate should be avoided and a diversity of herbicide sites-of-action must be maintained by using herbicide tank-mixtures or premixes with multiple effective sites-of-action, along with non-chemical weed control methods including crop rotation, tillage, competitive cultivars, weed seed destruction, and cover crops, among others (Norsworthy et al., 2012; Ganie and Jhala, 2017).

MON 87429 maize in combination with a deregulated glyphosate tolerance trait, such as NK603, will also enable use of quizalofop to control a broad spectrum of grass weed species in addition to non-selective herbicides, glufosinate and glyphosate. Quizalofop can be used either in mixture with glyphosate or in sequence with glyphosate or glufosinate to provide options for postemergence control of grass weed species. Many mixtures of graminicides including ACCase herbicides with glufosinate have been demonstrated to exhibit antagonism (Gardener et al., 2006; Burke et al., 2005). Either quizalofop or glufosinate applications on MON 87429 maize can provide control of glyphosate-resistant grass weeds and improve the control of annual and perennial grass weed species, some of which are difficult to control with glyphosate. Combinations of these application options may help delay potential evolution of resistance to quizalofop, glufosinate or glyphosate in grass species.

Dicamba, glufosinate, quizalofop and 2,4-D, as complementary herbicides to glyphosate, will provide additional weed control options in maize that strengthen the utility and sustainability of glyphosate as a weed control tool in glyphosate-tolerant maize systems. Likewise, glyphosate would be complementary to and strengthen the utility and sustainability of dicamba, glufosinate, quizalofop or 2,4-D as weed control tools for the combined commercial hybrid product containing MON 87429 and a deregulated glyphosate tolerance trait, such as NK603.

In the event there is known or suspected resistance to dicamba, glufosinate, quizalofop, 2,4-D, or glyphosate, or other herbicides, cultural and mechanical weed control options are available to the grower in the U.S. for managing resistant biotypes. There are multiple preplant and postemergence herbicide options (including soil residuals) for managing weed populations that are resistant or may potentially develop resistance to dicamba, glufosinate, quizalofop, 2,4-D or glyphosate in maize, as well for crops grown in rotation with maize. A representative short list of the effective herbicide options are noted in Table K-1 for a sub-set of common weed species in maize in the U.S., chosen based on if a weed species has biotype(s) that are resistant to herbicide(s) tolerated by MON 87429 maize (dicamba, glufosinate, 2,4-D and/or "fops") and biotypes of these species reported to have resistance to glyphosate either as multiple resistance (kochia, Palmer amaranth, Italian ryegrass) or resistance to glyphosate exist separately in biotypes with high potential for biotypes with combined resistance development (waterhemp, johnsongrass) (Heap, 2019; see Table VIII-6 and Appendix K.8). Table K-1, however, is

not intended to provide extensive set of weed control recommendations for any specific biotype(s) with unique cases of resistance present in a geography (county or state) that may include resistance to herbicide site(s)-of-action not tolerated by MON 87429 maize. Geography- and biotype-specific weed control recommendations can be obtained through state Cooperative Extension Service (CES) and/or through state-specific weed control guides published by the universities, where available (e.g., Kansas State University, 2019, Mississippi State University, 2019; University of Nebraska, 2019).

Table K-1. Representative Management Recommendations for Control of Common Weeds in Maize with Biotypes Resistant to Herbicide(s) Tolerated by MON 87429 and to Glyphosate

Weed Species[1,2]	Primary Crop	Rotational Crops			
	Maize	Soybeans	Cotton	Sorghum	Wheat
Kochia	Paraquat+ Atrazine[a] Isoxaflutole+ Atrazine[a] Thiencarbazone+ Tembotrione[a] Mesotrione[a]	Sulfentrazone+ Metribuzin[a] Flumioxazin+ Pyroxasulfone+ Chlorimuron[a] Saflufenacil+ Imazethapyr[a] Paraquat[a] Glufosinate[a,3]	Glufosinate[b,4]	Mesotrione+ s-Metolachlor + Atrazine[a] Atrazine[a] Acetochlor[a] Saflufenacil[a]	Carfentrazone+ 2,4-D[a] Triasulfuron+ Fluroxypyr[a] Pyrasulfotole+ Bromoxynil+ MCPA[a]
Tall Waterhemp	Topramezone[a] Saflufenacil[a] Isoxaflutole+ Atrazine[a] Mesotrione[a] Tembotrione[a] Glufosinate[a,4]	Sulfentrazone+ Metribuzin[a] Glyphosate+ Lactofen[a] Acetochlor[a] Flumioxazin[a] Fomesafen[a] Lactofen[a] Glufosinate[a,4]	Fomesafen[c] Diuron[c] Lactofen[c] Pyroxasulfone[c] +MSMA[c] Glufosinate[c,4]	Mesotrione+ s-Metolachlor + Atrazine[a] Saflufenacil[a] Acetochlor[a] Pyrasulfotole+ Bromoxynil[a]	Carfentrazone+ 2,4-D[a] Triasulfuron+ Fluroxypyr[a] Prosulfuron+ 2,4-D[a] Pyrasulfotole+ Bromoxynil+ 2,4-D[a]

Table K-1. Representative Management Recommendations for Control of Common Weeds in Maize with Biotypes Resistant to the Herbicide(s) Tolerated by MON 87429 Maize and to Glyphosate (continued)

Weed Species[1,2]	Primary Crop	Rotational Crops			
	Maize	Soybeans	Cotton	Sorghum	Wheat
Palmer amaranth	Acetochlor+ Atrazine[d] Thiencarbazone+ Isoxaflutole[d] Tembotrione+ Thiencarbazone[d] Bromoxynil+ Atrazine[d] Glufosinate[c,4]	Sulfentrazone+ Imazethapyr[d] Flumioxazin+ Pyroxasulfone[d] Lactofen[d] Glufosinate[c,4]	Fomesafen[c] Diuron[c] Pyroxasulfone + Carfentrazone[c] Glyphosate+ s-Metolachlor [c] Glufosinate[c,4]	s-Metolachlor + Mesotrione[d] Bromoxynil+ Atrazine[d] Carfentrazone[d]	Triasulfuron[d] Thifensulfuron+ Tribenuron+ Metsulfuron+ Dicamba[d] Bromoxynil+ MCPA[d]
Italian ryegrass	Carfentrazone+ Pyroxasulfone[c] Acetochlor+ Atrazine[c] Glyphosate[c] Pyroxasulfone[c]	Parquat[c] Glufosinate[c,4] Paraquat + Metribuzin[c] Paraquat + Oxyfluorfen[c]	Paraquat[c] Paraquat + Atrazine[c] Glyphosate + Saflufenacil[c] Glufosinate[c,4]	Paraquat[c] Paraquat+ Atrazine[c] Glyphosate + Saflufenacil[c, 3]	Thifensulfuron + Tribenuron[c] Pyroxasulfone[c] Mesosulfuron[c]

Table K-1. Representative Management Recommendations for Control of Common Weeds in Maize with Biotypes Resistant to the Herbicide(s) Tolerated by MON 87429 Maize and to Glyphosate (continued)

Weed Species[1,2]	Primary Crop	Rotational Crops			
	Maize	Soybeans	Cotton	Sorghum	Wheat
Johnsongrass	Glyphosate + s-Metolachlor + Mesotrione[c] Mesotrione + Rimsulfurone[c] Nicosulfurone[c] Glufosinate[c,4]	Glyphosate + s-Metolachlor [c] Glufosinate[c,4]	Trifloxysulfuron[c] Trifluralin[c] Glyphosate + s-Metolachlor [c] Glufosinate[c,4]	Glyphosate + Carfentrazone[c,3] Glyphosate + Flumioxazin[c,3] Paraquat + Atrazine[c]	Glyphosate + Carfentrazone[c,3] Glyphosate + Saflufenacil[c,3] Paraquat[c]

[1] Representative common weed species in maize in the U.S., chosen based on if a weed species has biotype(s) that is resistant to herbicide(s) tolerated by MON 87429 maize (dicamba, glufosinate, 2,4-D and/or "fops") and biotypes of these species reported to have resistance to glyphosate either as multiple resistance (kochia, Palmer amaranth, Italian ryegrass) or resistance to glyphosate exist separately in biotypes with high potential for biotypes with combined resistance development (waterhemp, johnsongrass) (Heap, 2019; see Table VIII-6 and Appendix K.8).
[2] Herbicide(s) represent a sub-set of recommendations only; unique case(s) of weed resistance in a state to be managed through local extension scientists and/or university weed control guides, if available.
[3] Glyphosate applied as burndown in non-glyphosate tolerant crops.
[4] Glufosinate applied on glufosinate-tolerant crops.
[a] University of Nebraska, 2019; [b] Take Action, 2016; [c] Mississippi State University, 2019; [d] Kansas State University, 2019;

K.11. Stewardship of Dicamba, Glufosinate, Quizalofop, 2,4-D and Glyphosate Use on MON 87429 Maize

To steward the use of agricultural herbicides and herbicide-tolerant cropping systems such as MON 87429 maize and the likely combined trait glyphosate-tolerant maize product, Monsanto has conducted investigations and worked extensively with academics and other herbicide registrants to understand and recommend best practices to manage herbicide resistance. These investigations have demonstrated that one of the major factors contributing to the development of resistant weed biotypes has been poor weed control management practices. The primary reasons for lack of adequate management includes: 1) application of herbicides at rates below those indicated on the product label for the weed species, and 2) sole reliance on a single herbicide for weed control without the use of other herbicides or cultural control methods (Beckie, 2006; Norsworthy et al. 2012).

K.11.1. Weed Control Recommendations

The proposed label for dicamba, and the labels for glufosinate, quizalofop, 2,4-D and glyphosate use on MON 87429 maize combined with deregulated glyphosate tolerance traits, such as NK603, are based on the maximum allowable use rates and patterns. Prior to launch of, for example, MON 87429 × NK603 maize, Monsanto, in cooperation with academics, will conduct trials to confirm the optimum rate and timing for dicamba, glufosinate, quizalofop, 2,4-D and glyphosate, alone and in combination with each other, and with other herbicides. Recommendations to growers will be developed from this information and will be provided in herbicide product labels, Monsanto's Technology Use Guide (TUG), and in other education and training materials to be broadly distributed. Specifically, research conducted by Monsanto showed the value of multiple soil-active preplant herbicides followed by an early postemergence application of a mixture of herbicides with multiple sites-of-action, including a soil active herbicide, against broadleaf weeds that included species in the genus *Amaranthus* (Bayer CropScience, 2019b). Such a program would optimize the likelihood of using two or more effective sites-of-action against the targeted weeds. In areas with glyphosate-resistant and hard to control broadleaf weed populations, dicamba or 2,4-D may be applied pre-planting as a tank mix with preemergence herbicides and/or applied post-emergence in addition to glufosinate in MON 87429 maize. In some situations, a second in-crop application of either dicamba or 2,4-D tank-mixed with glyphosate or glufosinate, with or without a soil residual herbicide will be recommended with options to rotate additional herbicides with diverse sites-of action between preplant and postemergence applications and as per the herbicide's label. For postemergence grass control, quizalofop application recommended alone or in combination with glyphosate or in sequence with glyphosate or glufosinate. In cases of managing both grass and broadleaf weed species, a time interval is to be considered between quizalofop application and any postemergence broadleaf herbicides as per the label recommendation (see Section VIII.F.3. for additional details).

These recommendations will encourage the use of more than one site-of-action against the targeted weed species, which is a fundamental component of a good weed resistance management program. These management systems, which include the use of multiple

effective herbicide sites-of-action, will reduce the potential for further resistance development to dicamba, glufosinate, 2,4-D, quizalofop and glyphosate as well as other critical maize herbicides.

K.11.2. Dispersal of Technical and Stewardship Information

Monsanto will use multiple methods to distribute technical and stewardship information to growers, academics and grower advisors. Monsanto's TUG (Monsanto, 2019) will set forth the requirements and best practices for cultivation of MON 87429 maize including recommendations on weed resistance management practices. Growers who purchase maize hybrids containing MON 87429 will be required to enter into a limited use license with Monsanto and must sign and comply with the Monsanto Technology Stewardship Agreement (MTSA), which requires the grower to follow the TUG.

The weed resistance management practices that will be articulated in the TUG will also be broadly communicated to growers and retailers in order to minimize the potential for development of resistant weed populations. These practices will be communicated through a variety of means, including direct mailings to each grower purchasing a maize hybrid containing MON 87429 and public websites[13]. The overall weed resistance management program will be reinforced through collaborations with U.S. academics, who will provide their recommendations for appropriate stewardship of dicamba, glufosinate, quizalofop, 2,4-D and glyphosate in maize production, as well as by collaboration with crop commodity groups who have launched web-based weed resistance educational modules. Finally, Monsanto will urge growers to report any incidence of non-performance of dicamba, glufosinate, quizalofop, 2,4-D or glyphosate on weeds in fields planted with MON 87429 maize to the manufacturer of the herbicide. Appropriate investigations of unsatisfactory weed control will be conducted.

The EPA is the U.S. federal regulatory agency that administers federal law governing pesticide sale and use under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). EPA encourages pesticide manufacturers to provide growers with information regarding an herbicide's site-of-action to aid growers in planning herbicide use practices and to foster the adoption of effective weed resistance management practices as specified by EPA in Pesticide Registration (PR) Notices 2017-1 and 2017-2 (U.S. EPA, 2017b; 2017c). The PR notice 2017-1 (U.S. EPA, 2017b) is a revised and updated version of an earlier PR notice 2001-5 (U.S. EPA, 2001), with much of its content being kept identical to the 2001-5 with the following changes: (1) provided additional guidance to registrants and a recommended format for resistance-management statements or information to place on labels; (2) included references to external technical resources on resistance management guidance; and (3) updated the instructions to submit changes to existing labels in order to enhance resistance-management language. The PR notice 2001-5 states that "this approach to resistance management is sound and would be highly beneficial to pesticide manufacturers and pesticide users." The EPA approves all pesticide label use

[13] http://tug.monsanto.com/, https://monsanto.com/products/product-stewardship/, http://www.roundupreadyplus.com/

instructions based on its evaluation of supporting data supplied by the pesticide registrant or manufacturer. By approving a label, EPA has concluded that the product will not cause unreasonable adverse effects to the environment when used in accordance with the label's directions. After EPA approves a pesticide label, it is a violation of federal law to use the pesticide for a use or in a manner not in accordance with the label directions. Monsanto incorporates EPA's guidelines for pesticide resistance management labeling on its agricultural herbicide labels and will continue to do so in the future.

In summary, Monsanto will require and educate weed resistance management practices through the Material Transfer Service Agreement (MTSA) and TUG for its biotechnology-derived herbicide-tolerant products, including MON 87429 maize. Upon deregulation, MON 87429 will be combined, via traditional breeding methods, with other maize traits (e.g., deregulated glyphosate tolerance traits, such as NK603), and Monsanto will promote these resistance management practices through product labeling and educational outreach efforts as an effective means to manage weed resistance development for dicamba, glufosinate, quizalofop, 2,4-D and glyphosate.

K.11.3. Weed Resistance Management Practices

Monsanto will provide information to growers and grower advisors on best management practices to delay development of additional resistance to dicamba, glufosinate, quizalofop and 2,4-D herbicides. Weed resistance management recommendations for the use of dicamba, glufosinate, quizalofop, 2,4-D and glyphosate herbicides in conjunction with maize hybrids containing MON 87429 will be consistent with the Herbicide Resistance Action Committee's guidelines for prevention and management of herbicide resistance (HRAC, 2015). These guidelines recommend an integrated approach to weed resistance management, including cultural, mechanical and chemical methods that include the use of multiple herbicide sites-of-action to manage a weed population.

In cases where resistance is confirmed for dicamba, glufosinate, quizalofop, 2,4-D or glyphosate in maize producing areas, Monsanto and University/Cooperative Extension Service (CES) personnel will provide recommendations for alternative herbicide control methods to growers. These recommendations can be made available through various channels including Monsanto supplemental labels, Monsanto and university publications, and internet sites to growers, consultants, retailers and distributors. For all existing cases of dicamba-, glufosinate-, quizalofop-, or 2,4-D- resistant weeds in the U.S. and globally today, alternative herbicides and cultural methods are available to growers to effectively control these biotypes. Examples of alternative herbicides from University/CES personnel that are applicable to weed species known to be resistant to dicamba and other synthetic auxin herbicides, glufosinate and "fops" herbicides are found in Table K-1. However, these examples in Table K-1 are only a representative subset of product combinations of available maize herbicides.

K.12. Monsanto Weed Performance Evaluation and Weed Resistance Management Plan

An important part of a weed resistance management plan is the timely acquisition of information regarding herbicide product performance. Monsanto has an extensive

technical, sales and marketing presence in maize markets where MON 87429 maize will be grown. Through our relationships with farm advisors, key University/CES personnel, and growers using our seeds and traits products, Monsanto will acquire important and timely information regarding product performance. This will allow the timely recognition of performance issues that could arise related to weed resistance or other means. Field employees and hired consultants are trained and provided processes for responding to product performance inquiries. Individual performance issues that could be related to potential resistance are promptly handled. In addition, performance inquiries are periodically reviewed by Monsanto for trends that could indicate the need for follow up action on a broad scale.

If new dicamba, glufosinate, quizalofop, 2,4-D or glyphosate resistance is confirmed, the scientific and grower communities will be notified, and a weed resistance management plan will be implemented by Monsanto in cooperation with the University/CES and/or the appropriate herbicide producer. The management plan will be designed to manage the resistant biotype through effective and economical weed management recommendations implemented by the grower. The plan considers what is technically appropriate for a particular weed and incorporates practical management strategies.

After a management plan is developed, Monsanto communicates the plan to the grower community through various means, that may include informational fact sheets, retailer training programs, agriculture media and/or other means, as appropriate.

K.13. Summary

Development of herbicide resistance in weeds is a complex process that can be difficult to accurately predict. Multiple methods for managing weed resistance are available and no single option is best for all farming situations. No single agronomic practice will mitigate resistance for all herbicides or all weeds. As a result, weed resistance needs to be managed on a case-by-case basis, tailored for the particular herbicide and weed species, and utilize an integrated system approach to meet grower needs. Using good weed management principles, built upon achieving high levels of control through proper application rate, choice of cultural and mechanical practices, and appropriate companion weed control products will allow dicamba, glufosinate, quizalofop, 2,4-D and glyphosate herbicides to continue to be used effectively. In cases where weed populations have evolved or developed resistance to dicamba, glufosinate, quizalofop, 2,4-D and/or glyphosate, effective management options are available, and experience has shown that growers will continue to find value in using dicamba, glufosinate, quizalofop, 2,4-D and glyphosate in their weed control programs.

The key principles for effective stewardship of dicamba, glufosinate, quizalofop, 2,4-D and glyphosate use comprise: a) Understand the biology of the weeds present; b) Use a diversified approach toward weed management focused on preventing weed seed production and reducing the number of weed seed in the soil seedbank; c) Plant into weed-free fields and then keep fields as weed free as possible; d) Plant weed-free crop seed; e) Scout fields routinely; f) Use multiple herbicide sites-of-action that are effective against the most troublesome weeds or those most prone to herbicide resistance; g) Apply

the labeled herbicide rate at recommended weed sizes; h) Emphasize cultural practices that suppress weeds by using crop competitiveness; i) Use mechanical and biological management practices where appropriate; j) Prevent field-to-field and within-field movement of weed seed or vegetative propagules; k) Manage weed seed at harvest and after harvest to prevent a buildup of the weed seedbank; l) Prevent an influx of weeds into the field by managing field borders

Overall, there are multiple factors that reduce the potential for dicamba or 2,4-D resistant broadleaf weed populations to arise from the use of dicamba or 2,4-D applied to MON 87429 combined with, for example, NK603. These are as follows:

- Dicamba or 2,4-D will be used in combination with glyphosate and/or glufosinate in a majority of cropping situations and weed recommendations will also include the concurrent use of residual herbicides for weed control and use of additional sites-of-action. These use patterns provide herbicides with multiple sites-of-action against major broadleaf weed species present in the maize production to delay the development of herbicide resistance.
- The development of resistance to auxin herbicides has been found to be relatively slow. This observation is hypothesized to be due to multiple sites-of-action within plants and evidence suggesting that resistance is determined by multiple genes (quantitative traits), at least in some species.
- Only two and five broadleaf weed species have been confirmed to be resistant to dicamba and 2,4-D, respectively, in the U.S., and relatively low numbers of broadleaf species have been confirmed to be resistant to synthetic auxin herbicides even though dicamba and 2,4-D have been widely in use for over 40 years.
- Known resistant broadleaf weed populations to dicamba, 2,4-D and other auxin herbicides are primarily found in the western U.S. and, have limited presence in the major maize geographies. Biotypes of problematic weeds resistant to 2,4-D were found in key maize growing states in the U.S. (for example, biotypes of waterhemp in Nebraska and Illinois and Palmer amaranth in Kansas). Known dicamba-resistant biotypes are not of major weed species present in the U.S. maize cropping systems. To delay resistance development in broadleaf weeds against dicamba or 2,4-D in the MON 87429 maize system, glufosinate can be used in combination and/or in sequence with 2,4-D or dicamba and in sequence with glyphosate, as allowed by the individual herbicide label. Residual herbicides will also be recommended and likely used in this cropping system.

Likewise, the probability for weed species to evolve resistance to glufosinate as a result of glufosinate use in the MON 87429 combined with glyphosate tolerance traits, such as NK603, is considered to be low because, only four species have been confirmed to be resistant to glufosinate worldwide and one (Italian ryegrass) in the U.S. This suggests that the frequency for resistant alleles in native weed populations is low. Known Italian ryegrass biotypes resistant to glufosinate herbicide within the U.S. are only found in Oregon and California, and thus, are not present in the major maize geographies. In the MON 87429 maize system, glufosinate can be used in combination and/or in sequence

with dicamba, quizalofop and/or 2,4-D and in sequence with glyphosate, as allowed by the individual herbicide label. Residual herbicides will also be recommended and likely used in this cropping system.

Resistance to quizalofop was reported in only five grass species in the U.S. Combining MON 87429 maize with glyphosate-tolerance traits, such as NK603, will also enable use of quizalofop to control a broad spectrum of grass weed species in addition to non-selective herbicides, glufosinate and glyphosate. Combination of these options will help delay or minimize potential evolution of resistance to quizalofop, glufosinate or glyphosate in grass species.

In the U.S., although resistance to glyphosate was reported in 17 weed species (11 broadleaf and 6 grass weed species), it is critical to maintain the efficacy of glyphosate and delay further development of glyphosate-resistant weed biotypes. MON 87429 maize when combined with other deregulated glyphosate-tolerant trait such as NK603, provides additional tools with multiple herbicide sites-of-action to manage glyphosate-tolerant weed biotypes and delay further glyphosate resistance development.

As noted above, MON 87429 maize when combined with other deregulated glyphosate tolerance traits, such as NK603, enable use of herbicides or herbicide mixtures with multiple sites-of-action to control broadleaf and grass weed species present in the maize production and can be used as part of an integrated weed management program with other SOAs (including residuals) and other weed control practices to delay development of herbicide resistant weed population.

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Appendix L: MON 87429 Exogenous Substrate Specificity

L.1. Purpose

The purpose of this study was to determine if MON 87429 FT_T enzyme could potentially bind and catabolize various herbicides applied exogenously. The five herbicides tested (Table L-1) were selected based on mode of action class and agronomic importance for controlling grassy weeds and volunteer corn. MON 87429 and the near isogenic conventional control, LH244 × HCL617, were planted in pots and randomly placed in a greenhouse. Fourteen (14) days after herbicide application, individual plants were visually rated for herbicide induced injury using a scale from 0 percent (no visible injury) to 100 percent (plant death). The results showed that MON 87429 had no damage when 2,4-D and quizalofop were tested as compared to the conventional control, but injury ratings for the other herbicides were similar between MON 87429 and the conventional control. Therefore, FT_T was able to reduce 2,4-D and quizalofop injury through catabolization of this herbicide, but is unlikely to have catabolized any of the other herbicides.

L.2. Materials

This study was conducted in a greenhouse at the Monsanto research facility in Chesterfield, MO in 2016. The starting seed for this study consisted of test material, MON 87429 (lot IHW300000879549985106881), and conventional maize control material, LH244 × HCL617 (lot IHW300000879550160526685), which has a genetic background similar to the test material.

The herbicides used in this study (Table L-1) are commercially available and were selected based on mode of action class and agronomic importance for controlling grassy weeds and volunteer corn. For each of the five herbicides applied, two application rates were used that are within the labeled range for each herbicide or known to cause injury to conventional maize.

L.3. Methods

The study was conducted in a greenhouse maintained at approximately 28° C during the day and 20° C during the night with a 16-hour photoperiod. Relative humidity was maintained between 30 and 70% while watering was conducted as needed.

The test and control starting seed were planted in 3.5 inch square pots with Redi-Earth® potting soil medium. Ten replicates of test and control seed were planted for each herbicide and rate combination and randomly placed in the greenhouse. At the 3-leaf growth stage, herbicide applications were made with a track sprayer according to standard practice. Visual injury ratings were assessed 14 days after herbicide application. A visual rating scale of 0-100% injury was used to rate the overall herbicide induced injury to each plant. A rating of 0% = no herbicide induced injury and 100% = plant death. Ratings were based on visual assessment of chlorosis, necrosis, malformation,

stunting, and biomass reduction. The ratings were averaged across 10 replications per treatment.

L.4. Results and Discussion

MON 87429 demonstrated no injury for 2,4-D and quizalofop when compared to the conventional control. MON 87429 and the conventional control exhibited similar injury ratings and therefore similar levels of susceptibility for all other herbicides tested (Table L-1). Clethodim, sethoxydim, and paraquat are important herbicides for grassy weed and volunteer maize control and will be effective in controlling volunteer MON 87429.

L.5. Conclusions

These results demonstrate that MON 87429 FT_T has a high specificity for 2,4-D and quizalofop as a substrate. Applications of various herbicides to MON 87429 and the near isogenic conventional control demonstrated that the rate of injury was similar for all of the herbicides tested, except 2,4-D and quizalofop, demonstrating only 2,4-D and quizalofop served as substrates for the FT_T present in MON 87429. Therefore, MON 87429 FT_T is specific for its substrates and did not catabolize other herbicides tested.

Table L-1 Induced Herbicide Injury to MON 87429 and Control

					Herbicide Induced Injury Ratings (%)[2]	
Formulation	Manufacturer	Herbicide	Labeled Rate Range[1] (g a.i./ha)	Rate Applied (g a.i./ha)	Control Mean (Range)[3]	MON 87429 Mean (Range)
Assure II®	DuPont	quizalofop	30.8-92.4	45	100 (100-100)	0 (0-0)
Assure II®	DuPont	quizalofop	30.8-92.4	90	100 (100-100)	0 (0-0)
2,4-D Amine 4	Helena	2,4-D	280-4480	840	30.5 (25-35)	0 (0-0)
2,4-D Amine 4	Helena	2,4-D	280-4480	1680	76.5 (50-90)	0 (0-0)
Select Max®	Valent	clethodim	110-280	140	100 (100-100)	99.9 (99-100)
Select Max®	Valent	clethodim	110-280	280	100 (100-100)	100 (100-100)
Poast®	BASF	sethoxydim	112-448	224	98.5 (98-100)	97.8 (95-99)
Poast®	BASF	sethoxydim	112-448	448	99.2 (98-100)	99.1 (98-100)
Gramoxone®	Syngenta	paraquat	340-530	265	49.0 (40-60)	47.5 (40-55)
Gramoxone®	Syngenta	paraquat	340-530	530	93.9 (80-100)	81.0 (70-90)

[1] Labeled rate range based on U.S. herbicide EPA approved labeled rates.
[2] Rated on a 0-100% injury scale where 0% = no herbicide induced injury and 100% = plant death. Ratings based on visual assessment of chlorosis, necrosis, malformation, stunting, and biomass reduction.
[3] Means calculated based on 10 replications per treatment.

Appendix M: Materials and Methods for Compositional Analysis of Maize MON 87429 Grain and Forage

Compositional comparisons between MON 87429 and the conventional control maize hybrid were performed using the principles and analytes outlined in the OECD consensus document for maize composition (OECD, 2002). These principles are accepted globally and have been employed previously in assessments of maize products derived through biotechnology. The compositional assessment was conducted on grain and forage samples harvested from multiple U.S. field sites during 2017 grown under typical agronomic field conditions practices.

M.1. Materials

Harvested grain and forage from MON 87429 and a conventional control that has similar genetic background to that of MON 87429 hybrid were compositionally assessed.

M.2. Characterization of the Materials

The identities of MON 87429 and the conventional control were confirmed prior to use in the compositional assessment.

M.3. Field Production of the Samples

Grain and forage samples were harvested from MON 87429 and the conventional control grown in the United States during the 2017 season. The field production was conducted at five sites. The field sites were planted in a randomized complete block design with four replicates per site. MON 87429 and the conventional control were grown under normal agronomic field conditions for their respective growing regions. MON 87429 plots were treated with dicamba, glufosinate, quizalofop and 2,4-D to generate samples under conditions of the intended use of the product.

Grain was harvested at physiological maturity and shipped at ambient temperature from the field sites to Monsanto Company. Forage was harvested at R5 and shipped on dry ice from the field sites to Monsanto Company. A subsample for compositional analysis was obtained from each tissue sample collected. These subsamples were ground and stored in a freezer set to maintain –20°C until their shipment on dry ice to Covance Laboratories Inc. (Madison, Wisconsin) for analysis.

M.4. Summary of Analytical Methods

Nutrients analyzed in this study included protein, total fat, ash, amino acids, linoleic acid, carbohydrates by calculation, fiber (acid detergent fiber (ADF), neutral detergent fiber (NDF)) in the grain, and protein, total fat, ash, carbohydrates by calculation and fiber (ADF and NDF) in the forage. The anti-nutrients assessed in grain included phytic acid and raffinose.

All compositional analyses were performed at Covance Laboratories, Inc. (Madison, Wisconsin). Methods for analysis were based on internationally-recognized procedures and literature publications. Brief descriptions of the methods utilized for the analyses are described below.

M.5. Acid Detergent Fiber

Sample aliquots were weighed into pre-weighed filter bags. Samples were placed in an ANKOM Fiber Analyzer and treated with an acid detergent solution containing sulfuric acid with cetyl trimethylammonium bromide, then filtered to remove proteins, starches, simple sugars, pectins, and ash. Fats and pigments were removed via an acetone wash leaving cellulose and lignin fractions. The remaining residue was the acid detergent fiber and was determined gravimetrically. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 1.00%.

M.6. Amino Acids

The following 10 amino acids were analyzed:

Total arginine	Total methionine
Total histidine	Total phenylalanine
Total isoleucine	Total threonine
Total leucine	Total tryptophan
Total lysine	Total valine

The samples were hydrolyzed in 6N hydrochloric acid for approximately 24 hours at approximately 106-118ºC. Tryptophan was hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for approximately 20 hours.

The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection. The amino acids were quantified using external standards. The results are reported on a fresh weight basis. The limit of quantitation for this study was 0.100 mg/g.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
L-Arginine Monohydrochloride	Sigma-Aldrich	SLBP2435V	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	SLBQ0900V	100
L-Isoleucine	Sigma-Aldrich	SLBF7068V	100
L-Leucine	Sigma-Aldrich	SLBQ0257V	99
L-Lysine Monohydrochloride	Sigma-Aldrich	SLBL2974V	99
L-Methionine	Sigma-Aldrich	SLBL7822V	100
L-Phenylalanine	Sigma-Aldrich	MKBQ7887V	99
L-Threonine	Sigma-Aldrich	SLBR2767V	100
L-Valine	Sigma-Aldrich	SLBS4748	100
L-Tryptophan	Sigma-Aldrich	SLBR5743V	100

M.7. Ash

All organic matter was driven off when the samples were ignited at approximately 550ºC in a muffle furnace for at least 5 hours. The remaining inorganic material was determined gravimetrically and referred to as ash. The results are reported on a fresh weight basis. The limit of quantitation was 0.100%.

M.8. Carbohydrate

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation:

% carbohydrates = 100 % - (% protein + % fat + % moisture + % ash)

The limit of quantitation was calculated as 0.100% on a fresh weight basis.

M.9. Fat by Acid Hydrolysis

The samples were hydrolyzed with hydrochloric acid. The fat was extracted using ether and hexane. The extracts were dried down and filtered through a sodium sulfate column. The remaining extracts were then evaporated, dried, and weighed. The limit of quantitation was calculated as 0.100% on a fresh weight basis.

M.10. Fat by Soxhlet Extraction

The samples were weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the samples to remove the fat. The extract was then evaporated, dried, and weighed. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.100%.

M.11. Linoleic Acid

The lipid was extracted and saponified with 0.5N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The results were converted to their triglyceride equivalent and reported on a fresh weight basis. The Methyl Linoleate analytical reference standard was purchased from Nu-Chek Prep. The lot numbers were N25-Z, AU31-A, F1-B and AU31A with a purity of 99.8%. The limit of quantitation was calculated as 0.00220%.

M.12. Moisture

The samples were dried in a vacuum oven at approximately 100°C. The moisture weight loss was determined and converted to percent moisture. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.100%.

M.13. Neutral Detergent Fiber

Sample aliquots were weighed into pre-weighed filter bags. Samples were placed in an ANKOM Fiber analyzer and treated with a neutral detergent solution containing EDTA. Samples were then filtered to remove proteins, simple sugars, pectins, and ash. Fats and pigments were removed via an acetone wash leaving hemicellulose, cellulose, and lignin fractions. Starches were removed with a heat stable alpha amylase soak. The remaining residue was the neutral detergent fiber and was determined gravimetrically. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 1.00%.

M.14. Phytic Acid

The samples were extracted using hydrochloric acid and sonication, purified using a silica-based anion exchange column, concentrated and injected onto a high-performance liquid chromatography (HPLC) system with a refractive index detector. The Phytic Acid Sodium Salt Hydrate analytical reference standard was purchased from Sigma-Aldrich. The lot number was BCBQ7037V with a purity of 78.280%. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.125%.

M.15. Protein

The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. Instrumentation was used to automate the digestion, distillation and titration processes. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.100%.

M.16. Raffinose

Sugars in the samples were extracted with a 50:50 water: methanol solution. Aliquots were taken, dried under inert gas, and then reconstituted with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucopyranoside as the internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid treatment, and then analyzed by gas chromatography using a flame ionization detector. The D-(+)-Raffinose pentahydrate analytical reference standard was purchased from Sigma-Aldrich. The lot number was WXBB7085V with a purity of 99.7%. The results are reported on a fresh weight basis. The limit of quantitation was calculated as 0.0500%.

M.17. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company for review. Data were then transferred to the Monsanto Regulatory Statistics Team where they were converted into

the appropriate units and statistically analyzed. The following formulas were used for re-expression of composition data for statistical analysis (Table M-1):

Table M-1. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Proximates, Fiber, Phytic Acid, Raffinose	% fw	% dw	X/d
Amino Acids (AA)	mg/g fw	% dw	X/(10d)
Fatty Acids (FA), Linoleic Acid	% fw	% Total FA	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA
[1]'X' is the individual sample value; d is the fraction of the sample that is dry matter.			

In order to complete a statistical analysis for a compositional constituent in this compositional assessment, at least 50% of all the values for an analyte in grain or forage had to be greater than the assay limit of quantitation (LOQ). No analytes with more than 50% of observations below the assay LOQ were observed.

The following linear mixed model was used for the combined-site analysis.

$$Y_{ijk} = \mu + S_i + R(S)_{j(i)} + M_k + (SM)_{ik} + \varepsilon_{ijk} \quad (1)$$

where:

Y_{ijk} is the observed response for the k_{th} substance in the j_{th} replicate of the i_{th} site;

μ is the overall mean;

S_i is the random effect of the i_{th} site;

$R(S)_{j(i)}$ is the random effect of the j_{th} replicate nested with the i_{th} site;

M_k is the fixed effect of the k_{th} substance;

$(SM)_{ik}$ is the random effect of the interaction between the i_{th} site and k_{th} substance;

ε_{ijk} is the residual error.

SAS PROC MIXED was used to fit model (1) separately for each component to conduct the statistical analysis. Studentized residuals were obtained to detect potential outliers in the dataset. Studentized residuals tend to have a standard normal distribution when

outliers are absent. Thus, most values are expected to be between ± 3. Data points that are outside of the ± 6 studentized residual ranges are considered as potential outliers. No value had a studentized residual outside of the ± 6 range for this study.

The linear mixed model (1) assumes that the experimental errors, ε_{ijk}, are independent, normally-distributed, and have a common variance. In this analysis, independence of the errors was controlled by the randomized complete block design. The normality and common variance assumptions were checked by visual examination of residual plots and histograms. No extreme violations were observed for any characteristic. Comparisons between MON 87429 and conventional control were defined within the model (1) and tested using t-tests.

References for Appendix M

OECD. 2002b. Consensus document on compositional considerations for new varieties of maize (*Zea mays*): Key food and feed nutrients, anti-nutrients and secondary plant metabolites. OECD ENV/JM/MONO (2002)25. Organisation of Economic Co-Operation and Development, Paris, France.

Appendix N: Digestive Fate of the FT_T Protein

N.1. Digestive Fate of the FT_T Protein

As part of a comprehensive evaluation, introduced proteins in biotechnology-derived crops are evaluated for their safety for human and animal consumption. Proteins are an essential dietary component for humans and animals, and most are rapidly degraded to the component amino acids for nutritional purposes (Hammond and Jez, 2011). Although the vast majority of ingested proteins are non-allergenic, a small set of proteins or their fragments have been associated with a variety of gastrointestinal and systemic manifestations of immune-mediated allergy. One characteristic of several food allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood et al., 1996; Moreno et al., 2005; Vassilopoulou et al., 2006; Vieths et al., 1999), although exceptions to the correlation can be identified (Fu et al., 2002). The enzymatic degradation of an ingested protein by exposure to gastric pepsin and/or intestinal pancreatic proteases (e.g., pancreatin) makes it highly unlikely that either the intact protein or protein fragment(s) will reach the absorptive epithelial cells of the small intestine where antigen processing cells reside (Moreno et al., 2005). Therefore, the susceptibility of FT_T to the presence of pepsin was evaluated using an assay protocol that has been standardized based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004).The susceptibility of proteins in the presence of pancreatin has also been used as a separate test system to assess the digestibility of food components (Okunuki et al., 2002; Yagami et al., 2000). The relationship between protein allergenicity and protein susceptibility to pancreatin degradation is limited, because the protein has not been first exposed to the acidic, denaturing conditions simulating the stomach, as would be the case with in vivo digestion (FAO-WHO, 2001).

N.1.1. Degradation of FT_T Protein in the Presence of Pepsin

Degradation of the FT_T protein by pepsin was evaluated over time by analyzing digestion mixtures incubated for targeted time intervals following a standardized protocol validated in an international, multi-laboratory ring study (Thomas et al., 2004) collected at targeted incubation time points. The specific methods used to assess the digestive fate/degradation of the FT_T protein in pepsin are summarized below and detailed in Appendix N.2. The study showed that the results of *in vitro* pepsin digestion assays using this protocol were reproducible and consistent for determining the digestive susceptibility of a protein. This standardized *in vitro* pepsin digestion protocol utilized a physiologically relevant acidic buffer appropriate for pepsin activity. The susceptibility of FT_T protein to pepsin degradation was assessed by visual analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel and by visual analysis of a western blot probed with an anti-FT_T polyclonal antibody. Both visualization methods were run concurrently with separate SDS-PAGE and western blot analyses to estimate the limit of detection (LOD) of the FT_T protein for each method.

For SDS-PAGE analysis of the digestibility of the FT_T protein in pepsin, the gel was loaded with 1 µg of total test protein (based on pre-digestion protein concentrations) for each of the digestion samples (Figure N-1, Panel A). The SDS-PAGE gel for the digestibility assessment was run concurrently with a separate SDS-PAGE gel to estimate the LOD of the FT_T protein (Figure N-1, Panel B). The LOD of intact FT_T protein was approximately 3.1 ng (Figure N-1, Panel B, lane 8). Visual examination of SDS-PAGE data showed that the intact FT_T protein was digested within 0.5 min of incubation in pepsin (Figure N-1, Panel A, lane 5). Therefore, based on the LOD, more than 99.7% (100% - 0.3% = 99.7%) of the intact FT_T protein was digested within 0.5 -min of incubation in pepsin. Transiently-stable peptide fragments at ~4-kDa were observed throughout the course of the digestion. There is a slight decrease in the intact FT_T protein band intensity and generation of fragments greater than 20 kDa observed in the 60 min No Pepsin Control compared to the 0 min No Pepsin Control (Figure N-1, Panel A, lanes 12 and 3, respectively). This likely indicates that a small portion of FT_T protein is instable possibly due to either the acidic conditions or minor contaminants in the FT_T solution; however, the rapid degradation of the intact FT_T protein and the subsequent fragments in the pepsin degradation samples was due to the proteolytic activity of pepsin.

The 0 min No Test Protein Control and 60 min No Test Protein Control (Figure N-1, Panel A, lanes 2 and 13) demonstrated that the pepsin is stable throughout the experimental phase.

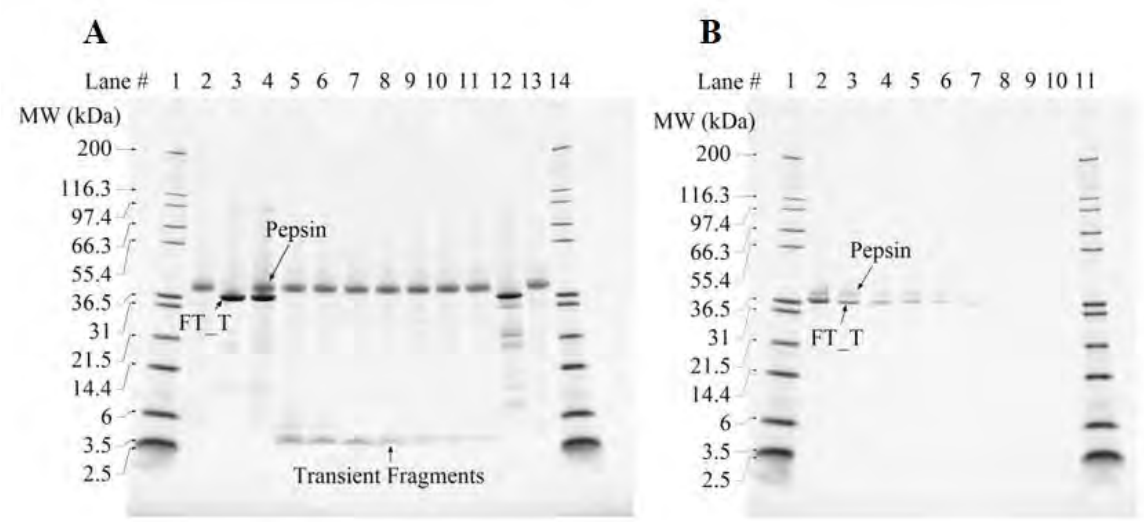


Figure N-1. SDS-PAGE Analysis of the Degradation of FT_T Protein by Pepsin

Colloidal Brilliant Blue G stained SDS-PAGE gels were used to assess the degradation of FT_T protein by pepsin. Molecular weights (kDa) are shown on the left of each gel and correspond to the markers loaded. In each gel, the FT_T protein migrated to approximately 35 kDa and pepsin to approximately 38 kDa. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images.

A: FT_T protein degradation in the presence of pepsin. Based on pre-reaction protein concentrations, 1 µg of test protein was loaded in each lane containing FT_T protein.

B: LOD determination. Indicated amounts of the test protein from the Pepsin Treated T0 sample were loaded to estimate the LOD of the FT_T protein.

Lane	Sample	Incubation Time (min)
1	Mark12 MWM	-
2	0 min No Test Protein Control	0
3	0 min No Pepsin Control	0
4	Pepsin Treated T0	0
5	Pepsin Treated T1	0.5
6	Pepsin Treated T2	2
7	Pepsin Treated T3	5
8	Pepsin Treated T4	10
9	Pepsin Treated T5	20
10	Pepsin Treated T6	30
11	Pepsin Treated T7	60
12	60 min No Pepsin Control	60
13	60 min No Test Protein Control	60
14	Mark12 MWM	-
15	Empty	-

Lane	Sample	Amount (ng)
1	Mark12 MWM	-
2	Pepsin Treated T0	200
3	Pepsin Treated T0	100
4	Pepsin Treated T0	50
5	Pepsin Treated T0	50
6	Pepsin Treated T0	25
7	Pepsin Treated T0	12.5
8	Pepsin Treated T0	3.1
9	Pepsin Treated T0	1.6
10	Pepsin Treated T0	0.8
11	Mark12 MWM	-
12	Empty	-
13	Empty	-
14	Empty	-
15	Empty	-

For western blot analysis of FT_T pepsin susceptibility, the FT_T protein was loaded with approximately 40 ng per lane of total protein (based on pre-reaction total protein concentrations) for each reaction time point examined. The western blot used to assess FT_T protein degradation (Figure N-2, Panel A) was run concurrently with the western blot used to estimate the LOD (Figure N-2, Panel B). The LOD of the FT_T protein was approximately 0.31 ng (Figure N-2, Panel B, Lane 10). Western blot analysis demonstrated that the intact FT_T protein was degraded below the LOD within 0.5 min of incubation in the presence of pepsin (Figure N-2, Panel A, Lane 6). Based on the western blot LOD for the FT_T protein, more than 99.2% (100% - 0.8% = 99.2%) of the intact FT_T protein was degraded within 0.5 min. No peptide fragments were detected at the 0.5 min and beyond time points in the western blot analysis.

No change in the intact FT_T protein band intensity were observed in the 0 min No Pepsin Control and the 60 min No Pepsin Control. There were three antibody-recognized fragments greater than 20 kDa observed in the 60 min No Pepsin Control that were not observed in the 0 min No Pepsin Control (Figure N-2, Panel A, lanes 13 and 4, respectively). This indicates that small portion of the FT_T protein is slightly instable possibly due to either the acidic conditions or minor contaminants in the FT_T solution; however, a majority of the degradation of the intact FT_T protein and the subsequent fragments in the pepsin degradation samples was due to the proteolytic activity of pepsin. The transiently-stable fragments at ~4 kDa that were observed by SDS-PAGE were not recognized by the antibody used in this western blot.

No immunoreactive bands were observed in 0 min No Protein Control and 60 min No Protein Control (Figure N-2, Panel A, lanes 3 and 14). This result indicates that there was no non-specific interaction between the pepsin solution and the FT_T-specific antibody under these experimental conditions.

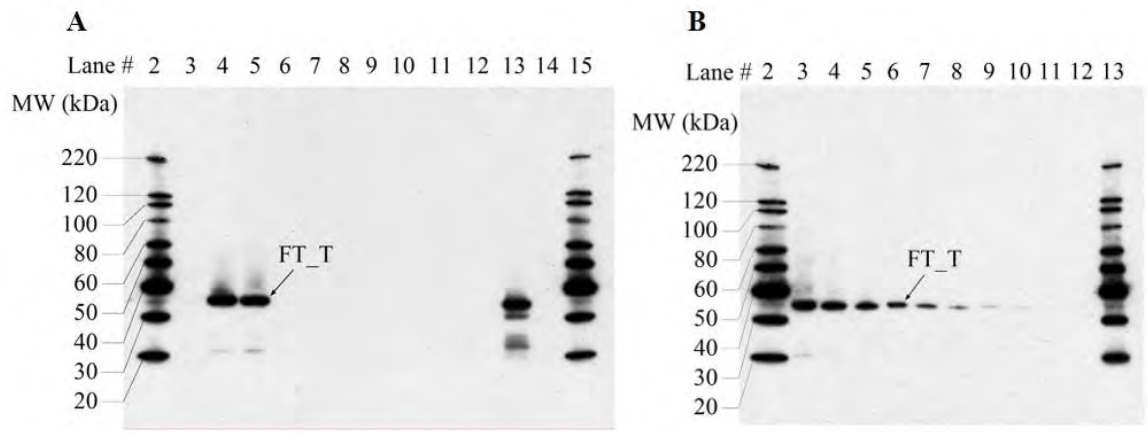


Figure N-2. Western Blot Analysis of the Degradation of FT_T Protein by Pepsin

Western blots probed with an anti-FT_T antibody were used to assess the degradation of FT_T by pepsin. Molecular weights (kDa) are shown on the left of each gel and correspond to the MagicMark™ molecular weight marker loaded in two lanes of each gel. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images. A 30 sec exposure is shown.

A: FT_T protein degradation by pepsin. Based on pre-reaction protein concentrations, 40 ng of test protein was loaded in each lane containing FT_T protein.

B: LOD determination. Indicated amounts of the test protein from the Pepsin Treated T0 sample were loaded to estimate the LOD of the FT_T protein.

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	0 min No Test Protein Control	0
4	0 min No Pepsin Control	0
5	Pepsin Treated T0	0
6	Pepsin Treated T1	0.5
7	Pepsin Treated T2	2
8	Pepsin Treated T3	5
9	Pepsin Treated T4	10
10	Pepsin Treated T5	20
11	Pepsin Treated T6	30
12	Pepsin Treated T7	60
13	60 min No Pepsin Control	60
14	60 min No Test Protein Control	60
15	MagicMark MWM	-

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	Pepsin Treated T0	40
4	Pepsin Treated T0	20
5	Pepsin Treated T0	10
6	Pepsin Treated T0	5
7	Pepsin Treated T0	2.5
8	Pepsin Treated T0	1.25
9	Pepsin Treated T0	0.63
10	Pepsin Treated T0	0.31
11	Pepsin Treated T0	0.16
12	Pepsin Treated T0	0.08
13	MagicMark MWM	-
14	Precision Plus MWM	-
15	Empty	-

N.1.2. Degradation of FT_T Protein in the Presence of Pancreatin

The degradation of the FT_T protein by pancreatin was assessed by western blot analysis (Figure N-3). The western blot used to assess the FT_T protein degradation (Figure N-3, Panel A) was run concurrently with the western blot used to estimate the LOD (Figure N-3, Panel B) of the FT_T protein. The LOD of the FT_T protein was observed at approximate 0.31 ng protein loading (Figure N-3, Panel B, lane 10). The LOD was used to calculate the maximum relative amount of FT_T protein that could remain visually undetected after digestion, which corresponded to approximately 0.8% of the total protein loaded.

The gel used to assess degradation of the FT_T protein by western blot was loaded with approximately 40 ng per lane of total protein (based on pre-reaction protein concentrations) for each reaction time point examined. Western blot analysis demonstrated that a band corresponding to the FT_T protein was degraded to a level below the LOD within 5 minutes of incubation with pancreatin (Figure N-3, Panel A, lane 5), the first time point assessed. Therefore, based on the LOD, more than 99.2% (100% - 0.8% = 99.2%) of the FT_T protein was digested within 5 minutes. No peptide fragments were detected at the 5 min and beyond time points in the western blot analysis.

No obvious change in the intact FT_T (~35.5 kDa) band intensity was observed in the absence of pancreatin in the 0 min No Pancreatin Control and 24 hour No Pancreatin Control (Figure N-3, Panel A, lanes 3 and 13). This indicates that the degradation of all immunoreactive forms of the FT_T protein was due to the proteolytic activity of pancreatin and not due to instability of the protein when incubated in 50 mM KH_2PO_4, pH 7.5 over the course of the experiment.

No immunoreactive bands were observed in the 0 min No Test Protein Control and 24 hour No Test Protein Control (Figure N-3, Panel A, lanes 2 and 14), demonstrating the absence of non-specific antibody interactions with the pancreatin solution.

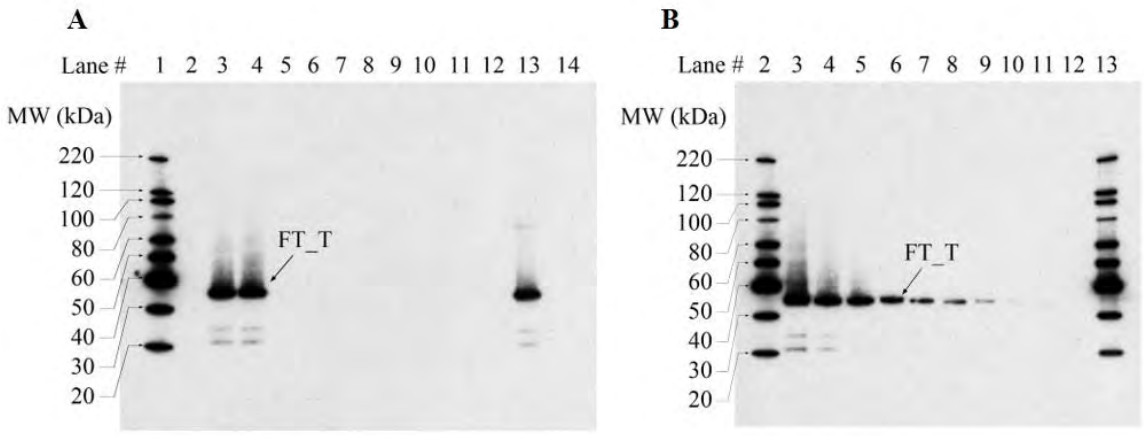


Figure N-3. Western Blot Analysis of the Degradation of FT_T Protein by Pancreatin

Western blots probed with an anti-FT_T antibody were used to assess the degradation of FT_T by pancreatin. Molecular weights (kDa) are shown on the left of each gel and correspond to the MagicMark™ molecular weight marker loaded in each gel. Empty lanes and molecular weight markers that were not visible on the film were cropped from the images. A 60 sec exposure is shown.

A: FT_T protein degradation by pancreatin. Based on pre-reaction protein concentrations, 40 ng of test protein was loaded in each lane containing FT_T protein.

B: LOD determination. Indicated amounts of the test protein from the Pancreatin Treated T0 sample were loaded to estimate the LOD of the FT_T protein.

Lane	Sample	Incubation Time
1	MagicMark MWM	-
2	0 min No Test Protein Control	0
3	0 min No Pancreatin Control	0
4	Pancreatin Treated T0	0
5	Pancreatin Treated T1	5 min
6	Pancreatin Treated T2	15 min
7	Pancreatin Treated T3	30 min
8	Pancreatin Treated T4	1 h
9	Pancreatin Treated T5	2 h
10	Pancreatin Treated T6	4 h
11	Pancreatin Treated T7	8 h
12	Pancreatin Treated T8	24 h
13	24 h No Pancreatin Control	24 h
14	24 h No Test Protein Control	24 h
15	Precision Plus MWM	-

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	MagicMark MWM	-
3	Pancreatin Treated T0	40
4	Pancreatin Treated T0	20
5	Pancreatin Treated T0	10
6	Pancreatin Treated T0	5
7	Pancreatin Treated T0	2.5
8	Pancreatin Treated T0	1.25
9	Pancreatin Treated T0	0.63
10	Pancreatin Treated T0	0.31
11	Pancreatin Treated T0	0.16
12	Pancreatin Treated T0	0.08
13	MagicMark MWM	-
14	Precision Plus MWM	-
15	Empty	-

N.1.3. Degradation of FT_T Protein by Pepsin Followed by Pancreatin

To better understand the fate of the transiently-stable peptide fragments at ~4 -kDa that were observed in the reaction mixtures throughout the course of the pepsin digestion of FT_T, sequential digestibility of the FT_T protein was conducted. This sequential digestibility was assessed both by visual analysis of a Colloidal Brilliant Blue G stained SDS-PAGE gel, and visual analysis of a western blot probed with an anti FT_T-polyclonal antibody. Methods for the sequential digestibility are provided in Appendix E.

For the sequential degradation assay, the FT_T protein was incubated with pepsin for 2 min, followed by incubation with pancreatin. For the Colloidal Brilliant Blue G stained SDS-PAGE assessment, the gel was loaded with 1 µg of FT_T protein (based on pre-digestion protein concentrations) for each of the digestion samples. Examination of SDS-PAGE data showed that the intact FT_T protein was digested within 2 min of incubation in pepsin (Figure N-4, Panel A, lane 3) and the small transient fragments at ~4 kDa was completely digested within 0.5 min of pancreatin exposure (Figure N-4, Panel A, lane 7).

No change in the fragment band intensities was observed in the absence of pancreatin in the SEQ 0 min No Pancreatin Control and SEQ 2 hour No Pancreatin Control (Figure N-4, Panel A, lanes 5 and 14). This indicates that the digestion of the fragments was due to the proteolytic activity of pancreatin and not due to instability of the fragment when incubated in 50 mM KH_2PO_4 at pH 7.5 over the course of the experiment.

The SEQ 0 min No Test Protein Control and SEQ 2 hour No Test Protein Control (Figure N-4, Panel A, lanes 4 and 15) demonstrated the integrity of the pancreatin over the course of the experiment. The intensity of some pancreatin bands decreased somewhat during the course of the experiment, most likely due to auto-digestion. This is not expected to adversely impact the pancreatin degradation results, as the transiently stable fragments were digested within 0.5 min of exposure to pancreatin.

The sequential digestion of the FT_T protein was also assessed by western blot (Figure N-4, Panel B), with 40 ng of the test protein (based on total protein pre-digestion concentrations) loaded per lane. No bands were detected in the 2 min Pepsin Treated sample (Figure N-4, Panel B, lane 3).

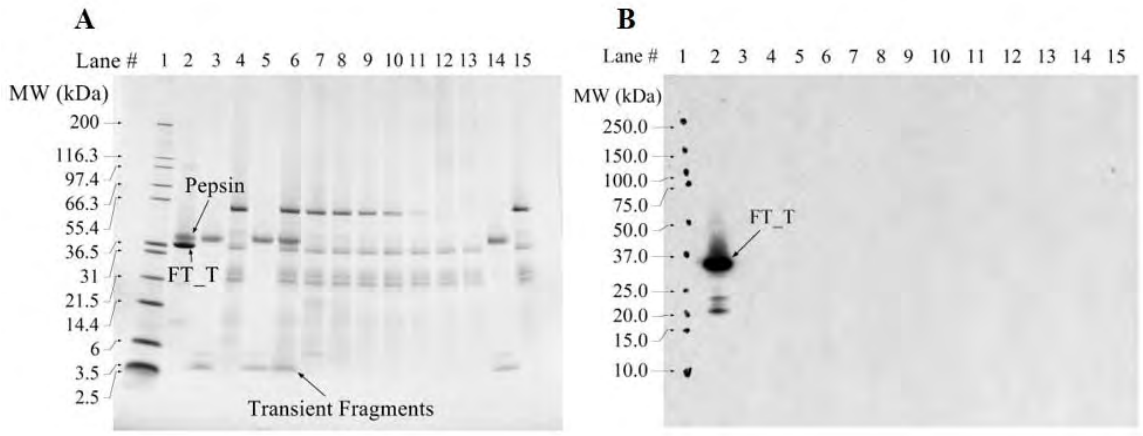


Figure N-4. SDS-PAGE and Western Blot Analysis of the Degradation of FT_T Protein by Sequential Digestion

SDS-PAGE and western blot analysis were used to assess the degradation of FT_T in sequential digestion. Molecular weights (kDa) are shown on the left of each gel and correspond to the markers loaded (cropped in panel B).

A: Colloidal Brilliant Blue G stained SDS-PAGE gel analysis of FT_T in sequential digestion. Based on pre-digestion protein concentrations, 1 µg of test protein was loaded in each lane containing FT_T protein.

B: Western blot analysis of FT_T in sequential digestion. Based on pre-digestion protein concentrations, 40 ng of test protein was loaded in each lane containing FT_T protein. A 60 sec exposure is shown.

Lane	Sample	Incubation Time
1	Mark12 MWM	-
	Pepsin Degradation	
2	0 min Pepsin Treated	0 min
3	2 min Pepsin Treated	2 min
	Pancreatin Degradation	
4	SEQ 0 min No Test Protein Control	0 min
5	SEQ 0 min No Pancreatin Control	0 min
6	SEQ T0	0 min
7	SEQ T1	0.5 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ 2 h No Pancreatin Control	2 h
15	SEQ 2 h No Test Protein Control	2 h

Lane	Sample	Incubation Time
1	Precision Plus MWM	-
	Pepsin Degradation	
2	0 min Pepsin Treated	0 min
3	2 min Pepsin Treated	2 min
	Pancreatin Degradation	
4	SEQ 0 min No Test Protein Control	0 min
5	SEQ 0 min No Pancreatin Control	0 min
6	SEQ T0	0 min
7	SEQ T1	0.5 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ 2 h No Pancreatin Control	2 h
15	SEQ 2 h No Test Protein Control	2 h

N.1.4. Degradation of FT_T Protein Conclusion

The ability of FT_T protein to be degraded by pepsin and by pancreatin was evaluated in this study. The results showed that at least 99.7% of the intact FT_T protein was degraded by pepsin within 0.5 min when analyzed by SDS-PAGE and at least 99.2% of the intact FT_T was degraded by pepsin within 0.5 min when analyzed by western blot using a FT_T specific antibody. SDS-PAGE analysis showed that transient peptide fragments at ~4 kDa were observed throughout the course of the pepsin digestion. At least 99.2% of the intact FT_T protein was degraded by pancreatin within 5 min when analyzed by western blot. These results show that the full-length FT_T is rapidly degraded by pepsin and pancreatin. The transient fragments at ~4 kDa were rapidly degraded by sequential digestion, indicating that gastrointestinal digestion is sufficient to degrade the intact FT_T protein and any fragments thereof. Rapid and complete degradation of the FT_T protein by pancreatin alone and pepsin followed by pancreatin indicates that the FT_T protein poses no meaningful risk to human or animal health.

N.2. Materials and Methods Used in Assessing Stability of FT_T Proteins in Pepsin and Pancreatin

N.2.1. Test Substance

The test substance was the FT_T protein (lot # 11478164) purified from *Escherichia coli* (*E. coli*) transformed with the pMON374248 plasmid (*E. coli*-produced FT_T, referred to in this document as FT_T protein). The FT_T protein is stored in a -80 °C freezer in a buffer solution containing 40 mM Tris, pH 8, 0.2 M NaCl (storage buffer).

N.2.2. Characterization of Test Substance

The test protein (lot 11478164) had a purity of 92%, a total protein concentration of 4.8 mg/ml, and an apparent molecular weight of 35.5 kDa.

N.3. Experimental Design

Assays designed to assess the degradation of FT_T protein by pepsin and by pancreatin were performed independently. Because protein fragments attributable to the FT_T protein were identified after 10 min of pepsin degradation, a sequential digestion of the FT_T protein with pepsin followed by pancreatin was also performed.

N.3.1. Preparation of the Pepsin Stock Solution

High purity pepsin (Sigma catalog number P6887; specific activity of 3546 U/mg, 96% protein, purity-corrected specific activity of 3404 U/mg) was dissolved in 10 mM HCl, 2 mg/ml NaCl, pH ~1.2 to a concentration of ~2,632 U/ml. The amount of powder used to prepare the pepsin stock solution was calculated by using the purity-corrected pepsin specific activity value of 3404 U/mg. Activity was assessed using a pepsin activity assay (Section N.4.1).

N.3.1.1. Degradation of the FT_T Protein by Pepsin

Degradation of the FT_T protein by pepsin was evaluated over time by analyzing samples collected at targeted incubation time points. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
0 min	0 min No Test Protein Control
0 min	0 min No Pepsin Control
0 min	Pepsin Treated T0
0.5 min	Pepsin Treated T1
2 min	Pepsin Treated T2
5 min	Pepsin Treated T3
10 min	Pepsin Treated T4
20 min	Pepsin Treated T5
30 min	Pepsin Treated T6
60 min	Pepsin Treated T7
60 min	60 min No Pepsin Control
60 min	60 min No Test Protein Control

The reaction mixture was prepared by adding 911.9 µl of pre-heated (37.2ºC, 10 min) pepsin stock solution to a tube containing 50 µl of FT_T protein, which corresponded to 240 µg of FT_T protein and 2400 U of pepsin. The tube contents were mixed and immediately returned to the water bath (37.1ºC). Samples (96.2 µl) were removed at 0.5, 2, 5, 10, 20, 30 and 60 min (corresponding to Pepsin Treated T1 through Pepsin Treated T7). Each 96.2 µl sample was placed immediately in a tube containing quenching mixture consisting of 33.7 µl of 0.7 M sodium carbonate buffer and 32.5 µl of 5× loading buffer (5× LB; ~310 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8).

The Pepsin Treated T0 sample was prepared in a separate tube. The pepsin stock solution (91.2 µl) was quenched by the addition of 0.7 M sodium carbonate buffer (33.7 µl) and 5× LB (32.5 µl) and heated at 95-100 ºC for 5-10 min prior to the addition of the FT_T protein (5.0 µl).

All quenched samples were frozen on dry ice and stored in a 80 ºC freezer until analysis.

N.3.1.2. Pepsin Degradation Experimental Controls

Experimental control samples were prepared to determine the stability of the FT_T protein in 10 mM HCl, 2 mg/ml NaCl, pH ~1.2, the solution used to prepare the pepsin stock solution. The 0 min No Pepsin Control was prepared in a similar manner as described in Section N.4.1 for Pepsin Treated T0, except that 10 mM HCl, 2 mg/ml NaCl, pH 1.2 was added instead of the pepsin stock solution. The 60 min No Pepsin Control was prepared in a manner similar to 0 min No Pepsin Control, except the protein and

10 mM HCl, 2 mg/ml NaCl, pH 1.2 were incubated for 60 min at 37.1ºC before quenching with 0.7 M sodium carbonate buffer and 5× LB.

Experimental control samples were also prepared to determine the stability of the pepsin stock solution lacking the FT_T protein. The 0 min No Test Protein Control was prepared in a similar manner as described in Section N.4.1 for Pepsin Treated T0, except that protein storage buffer (40 mM Tris, pH 8, 0.2 M NaCl) was added in place of the FT_T protein. The 60 min No Test Protein Control was prepared in a manner similar to 0 min No Test Protein Control, except that protein storage buffer and the pepsin stock solution were incubated for 60 min at 37.1ºC before quenching with 0.7 M sodium carbonate buffer and 5× LB.

All quenched samples were frozen on dry ice and stored in a 80 ºC freezer until analysis.

N.3.2. Preparation of the Pancreatin Stock Solution

Pancreatin contains a mixture of proteolytic enzymes and was prepared based on the method described in The United States Pharmacopoeia (USP, 1995). The pancreatin was obtained from Alfa Aesar (catalog number J62162) and was dissolved in 50 mM potassium phosphate buffer (pH 7.5) to a concentration of 10 mg of pancreatin powder/ml. Activity was assessed using a pancreatin activity assay (Section N.4.2)

N.3.2.1. Degradation of the FT_T Protein by Pancreatin

Degradation of the intact FT_T protein by pancreatin was evaluated over time by analyzing samples at multiple incubation time points. A numerical code using the numbers 0 through 8 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
0 min	0 min No Test Protein Control
0 min	0 min No Pancreatin Control
0 min	Pancreatin Treated T0
5 min	Pancreatin Treated T1
15 min	Pancreatin Treated T2
30 min	Pancreatin Treated T3
1 h	Pancreatin Treated T4
2 h	Pancreatin Treated T5
4 h	Pancreatin Treated T6
8 h	Pancreatin Treated T7
24 h	Pancreatin Treated T8
24 h	24 h No Pancreatin Control
24 h	24 h No Test Protein Control

The reaction mixture was prepared by adding 929 µl of pre-heated (37.0ºC, 10 min) pancreatin stock solution to a tube containing 35 µl of the FT_T protein, corresponding to 168 µg of FT_T protein and 9,290.4 µg of pancreatin. The tube contents were vortex

mixed and immediately returned to the water bath (37.0ºC). Digestion samples (96.4 µl) were removed at 5, 15, 30 min, 1, 2, 4, 8, and 24 h (corresponding to time points Pancreatin Treated T1 through Pancreatin Treated T8) and immediately placed in a tube containing 24.1 µl of 5× LB, heated to 95-100 ºC for 5 10 min, and frozen on dry ice for complete quenching.

The Pancreatin Treated T0 sample was prepared in a separate tube by first quenching 92.9 µl of pancreatin stock solution with 24.1 µl of 5× LB buffer and heating to 95-100 ºC for 5 10 min prior to the addition of 3.5 µl of the FT_T protein.

All quenched samples were frozen on dry ice and stored in a 80 ºC freezer until analysis.

N.3.2.2. Pancreatin Degradation Experimental Controls

Experimental control samples were prepared to determine the stability of the FT_T protein in 50 mM KH2PO4, pH 7.5, the buffer used to prepare pancreatin stock solution. The 0 min No Pancreatin Control was prepared in a similar manner as described in Section N.4.2 for Pancreatin Treated T0, except that 50 mM KH2PO4, pH 7.5 was added instead of the pancreatin stock solution. The 24 hour No Pancreatin Control was also prepared in a similar manner, except the protein and 50 mM KH2PO4, pH 7.5 were incubated for 24 hour at 37.0ºC before quenching with 5× LB and heating.

Experimental control samples were also prepared to characterize the test system lacking the FT_T protein. The 0 min No Test Protein Control was prepared in a similar manner as described in Section N.4.2 for Pancreatin Treated T0, except that protein storage buffer (40 mM Tris, pH 8, 0.2 M NaCl) was added instead of FT_T protein. The 24 hour No Test Protein Control was also prepared in a similar manner, except the protein storage buffer and pancreatin stock solution were incubated for 24 hour at 37.0ºC before quenching with 5× LB and heating.

All quenched samples were frozen on dry ice and stored in a 80 ºC freezer until analyzed.

N.3.3. Degradation of the FT_T Protein by Pepsin Followed by Pancreatin

Degradation of the FT_T protein by sequential digestion was evaluated by first incubating the FT_T protein with pepsin for 2 min, quenching the pepsin degradation reaction, and then incubating the reaction mixture with pancreatin. The sequential digestion of the FT_T protein was evaluated over time by analyzing samples at multiple incubation time points with a numerical code using the number 0 through 7 to distinguish incubation time points as follows:

Targeted Incubation Time Point	Sample Code
Pepsin Degradation	
0 min	0 min Pepsin Treated
2 min	2 min Pepsin Treated
Pancreatin Degradation	
0 min	SEQ 0 min No Test Protein Control
0 min	SEQ 0 min No Pancreatin Control
0 min	SEQ T0
0.5 min	SEQ T1
2 min	SEQ T2
5 min	SEQ T3
10 min	SEQ T4
30 min	SEQ T5
1 h	SEQ T6
2 h	SEQ T7
2 h	SEQ 2 h No Pancreatin Control
2 h	SEQ 2 h No Test Protein Control

In the pepsin degradation phase, 729.5 µl of pre-heated (37.4ºC, 10 min) pepsin solution (2632 units of pepsin activity/ml) was added to 40 µl of the FT_T protein, corresponding to 192 µg of FT_T protein and 1920 U of pepsin. The tube contents were mixed by vortexing and immediately returned to the water bath (37.3ºC). The tube was removed at a targeted time of 2 min, and immediately quenched by adding 269.3 µl of 0.7 M sodium carbonate buffer. The 2 min Pepsin Treated sample was prepared by mixing 120 µl of the quenched sample with 30 µl of 5× LB, and heating to 95-100 ºC for 5-10 min. The sample was then frozen on dry ice and stored in a -80 °C freezer until analyzed.

In the pancreatin degradation phase, the quenched 2 min pepsin treated sample was digested in pancreatin. For this phase, 525.4 µl of pre-heated (37.3ºC) pancreatin solution was added to 500 µl of quenched 2 min pepsin treated sample, corresponding to 95 µg total FT_T protein (based on predigested concentration) and 5,253.5 µg of pancreatin. The tube contents were mixed by vortexing and immediately returned to the water bath (37.2ºC). Digestion samples (102.5 µl) were removed from the tube at targeted times of 30 sec, 2, 5, 10, 30 min, 1, and 2 h (corresponding to specimen codes SEQ T1 through SEQ T7) and immediately quenched by placing in a tube containing 25.6 µl of 5× LB, heated to 95-100 ºC for 5-10 min, and frozen on dry ice for complete quenching.

The zero incubation time point for the pepsin degradation phase (0 min Pepsin Treated) was prepared in a separate tube by first quenching 73 µl of pepsin solution with 26.9 µl of sodium carbonate buffer and 26 µl of 5× LB, then heating to 95-100 ºC for 5-10 min prior to the addition of 4 µl of the FT_T protein.

The zero incubation time point for the pancreatin digestion phase (SEQ T0) was prepared in a separate tube by first quenching 52.5 µl of pancreatin solution with 25.6 µl of 5× LB

buffer and heating to 95-100 ºC for 5-10 min prior to the addition of 50 µl of the quenched 2 min pepsin treated FT_T.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analyzed.

N.3.3.1. Pepsin Followed by Pancreatin Degradation Experimental Controls

The SEQ 0 min No Pancreatin Control was prepared in a similar manner as described for SEQ T0, except that 50 mM KH_2PO_4, pH 7.5 was added instead of pancreatin solution. The SEQ 2 hour No Pancreatin Control was also prepared in a similar manner, except the protein and 50 mM KH_2PO_4 were incubated for 2 hour at 37.2°C before quenching with 5× LB and heating.

The SEQ 0 min No Test Protein Control was prepared in a similar manner as described in for SEQ T0, except that protein storage buffer (40 mM Tris, pH 8, 0.2 M NaCl) was added instead of FT_T protein. The SEQ 2 hour No Test Protein Control was also prepared in a similar manner, except the protein storage buffer and pancreatin solution were incubated for 2 hour at 37.2ºC before quenching with 5× LB and heating.

All quenched samples were frozen on dry ice and stored in a -80 ºC freezer until analyzed.

N.4. Analytical Methods

N.4.1. Pepsin Activity Assay

Pepsin activity was determined by measuring the degradation of denatured hemoglobin. To conduct the assay, the pepsin stock solution (Section 4.1), was diluted to 0.03 mg of powder per ml with 10 mM HCl, 2 mg/ml NaCl, pH ~1.2 (dilution factor (DF) = 25.67). Five ml of acidified hemoglobin (2% (w/v)) was added to 3 test sample tubes and 3 blank tubes and all were pre-warmed for 8-10 min in a water bath at 37.0-37.1°C. Diluted pepsin stock solution (1 ml) was added to the 3 test sample tubes (test samples) and all 6 tubes were incubated for 10 min in the water bath. Ten ml of 5% (v/v) chilled trichloroacetic acid (TCA) was added to each of the 6 tubes and 1 ml diluted pepsin stock solution was then added to the 3 blank samples. Samples were mixed and then incubated for another 5-10 min in the water bath. Precipitated protein was removed from each sample using 0.45 µm syringe filters and the absorbance of the test and blank sample filtrates at 280 nm were measured using a SpectraMax M2 plate reader (Molecular Devices). One unit of pepsin produces a change in absorbance at 280 nm of 0.001 per min at 37 ± 2 ºC, pH 1.2. The units of pepsin in 1 ml of the stock solution were calculated using the following equation:

$$\frac{Mean\ Test_{A280nm} - Mean\ Blank_{A280nm}}{0.001\ \times\ 10\,\text{min}\ \times\ 1\,ml} \times DF$$

where 0.001 is the change in the absorbance at 280 nm per min produced by one unit of pepsin activity at 37 ± 2 ºC, pH 1.2; 10 min is the reaction time; 1 ml is the volume of the diluted pepsin stock solution added to the reaction; and DF is the dilution factor for the

pepsin stock solution. The activity of pepsin was converted from units/ml to units/mg. Acceptable specific activity (units/mg pepsin powder) for the pepsin solution was equal to the specific activity determined by the manufacturer ± 1,000 units/mg.

N.4.2. Pancreatin Activity Assay

The pancreatin activity was determined by measuring the degradation of resorufin-labeled casein (Roche Life Science). To conduct the assay, 50 µl of 0.4% (w/v) resorufin-labeled casein and 50 µl of incubation buffer (200 mM Tris, pH 7.8, 20 mM CaCl2) were added to 3 test sample tubes and 3 blank tubes and all were pre-warmed for 3 min in a water bath at 37.0-37.3°C. To initiate the reaction, 100 µl of 0.05× pancreatin stock solution (pancreatin stock solution was diluted to 0.05× with 50 mM KH2PO4, pH 7.5 before the activity assay was initiated) was added to each of the 3 test sample tubes while 100 µl of 50 mM KH2PO4, pH 7.5 was added to each of the 3 blank tubes. All 6 tubes were incubated for 15 min in the water bath. Reactions were quenched by adding 480 µl of 5% (w/v) TCA to each tube, followed by vortex mixing. The samples were incubated in the water bath for an additional 10 min. The supernatants recovered after centrifugation (400 µl) were neutralized by the addition of 600 µl assay buffer (500 mM Tris-HCl, pH 8.8), and the absorbance of the test and blank supernatants was read at 574 nm using a SpectraMax M2 plate reader (Molecular Devices). One unit of pancreatin produces a change in the absorbance at 574 nm of 0.001 per min at 37 ± 2 ºC. The units of pancreatin in the stock solution were calculated using the following equation:

$$\frac{Mean\ Activity_{A574nm} - Mean\ Blank_{A574nm}}{0.001\ \times 15\,\text{min} \times\ 0.005\ ml}$$

where 0.001 is the change in the absorbance at 574 nm per min at 37 ± 2 ºC produced by one unit of pancreatin activity, 15 min is the reaction time, 0.1 ml is the volume of diluted pancreatin stock solution added to the reaction, and 0.005 ml is the volume of the pancreatin stock solution used in the assay (0.05× Pancreatin solution, 0.1 ml). An acceptable specific activity for the pancreatin stock solution was defined as 11,000 ± 3,000 U/ml.

N.4.3. SDS-PAGE and Colloidal Brilliant Blue G Staining

Pepsin-treated samples and associated control samples were subjected to SDS-PAGE using pre-cast Tricine 10-20% (w/v) polyacrylamide gradient mini-gels and Tricine SDS running buffer (Invitrogen). The FT_T protein was loaded at 1 µg per lane based on total pre-digestion protein concentration. The experimental controls were loaded at the same volumes as those containing FT_T protein so that they would be comparable. Mark12™ molecular weight marker (Invitrogen) was loaded to estimate the relative molecular weight of proteins and peptides. Electrophoresis was performed at a constant voltage of 165 volts for 60-61 minutes. After electrophoresis, gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min and stained for 17-20 hours in 1× Brilliant Blue G-Colloidal stain solution (Sigma), destained for 30 sec in 10% (v/v) acetic acid, 25% (v/v) methanol and then destained for 4.25-6 hours in a 25% (v/v)

methanol solution. The gels were scanned using a Bio Rad GS 900 densitometer (Bio Rad) to produce digitized images to be used as figures for reporting purposes.

To estimate the LOD of the FT_T protein, dilutions of the Pepsin Treated T0 samples were loaded on a second SDS-PAGE gel and the gel was run and processed exactly as the gel used to assess FT_T protein degradation in the presence of pepsin. Loads per lane were approximately 200 to 0.8 ng of the FT_T. The LOD was determined as the lowest amount of the FT_T protein that was visible on the gel with Colloidal staining.

Sequential digestion samples were also analyzed by SDS-PAGE as described above for pepsin degradation samples.

N.4.4. Western Blot Analysis

Pepsin-treated, pancreatin-treated, and sequential digestion samples, and the associated control samples, were subjected to SDS-PAGE using pre-cast Tricine 10-20% (w/v) polyacrylamide gradient mini-gels. The FT_T protein was loaded at approximately 40 ng per lane based on total pre-digestion protein concentration. The experimental controls were loaded to equal the digestion samples. Electrophoresis was performed at a constant voltage of 165 volts for 60-70 minutes. After electrophoresis, the proteins were electrotransferred to PVDF membranes (Bio Rad). Pre-stained molecular weight markers (Precision Plus Protein™ Standards Dual Color Standard, Bio Rad; MagicMark™ XP, Invitrogen) were used to verify electrotransfer of the proteins to the membranes and estimate the relative molecular weight of proteins.

Proteins transferred to PVDF membranes were analyzed by western blot. The western blotting procedure was performed using an iBind™ western System apparatus (Life Technologies). The membranes were blocked with 1× iBind™ Solution (Life Technologies) and incubated with goat anti FT_T antibody (lot 27003) at a dilution of 1:1,000 in 1× iBind™ Solution. After washing with 1× iBind™ Solution, the membrane was next incubated with horseradish peroxidase (HRP)-conjugated horse anti goat IgG (Vector Laboratories) at a dilution of 1:1,500 in 1× iBind™ Solution and washed again with 1× iBind™ Solution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare) and exposed to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare). The film was developed using a Konica SRX 101A automated film processor (Konica). The films were scanned using a Bio Rad GS 900 densitometer to produce electronic images to be used as figures for reporting purposes.

To estimate the LOD of the FT_T protein, dilutions of either the Pepsin Treated T0 or the Pancreatin Treated T0 samples were subjected to western blot analysis run and processed exactly as the western blot used to assess FT_T protein degradation in the presence of pepsin or pancreatin, respectively. Loads per lane were approximately 40 to 0.08 ng of the FT_T protein. The LOD was determined as the lowest amount of the FT_T protein that was visible on the film.

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By USDA APHIS BRS Document Control Officer at 2:20 pm, Dec 11, 2019

Supplemental Information for Technical Completeness of Petition 19-316-01p (Monsanto Petition Number CR279-19U4; revised December 10, 2019)

Monsanto[1] is providing the following information as a supplement to Petition Number 19-316-01p (Monsanto Petition Number CR279-19U4) requesting the Determination of Nonregulated Status for Dicamba, Glufosinate, Quizalofop and 2,4-Dichlorophenoxyacetic Acid Tolerant MON 87429 Maize with Tissue Specific Glyphosate Tolerance Facilitating the Production of Hybrid Maize Seed (OECD Unique Identifier: MON 87429-9). This information also supports the clarifications requested in the Clarification Letter from USDA-APHIS dated November 20, 2019.

1. The cited 2018 reference has been marked as CBI in the three locations on pages 131, 136 and 181.

   A brief description of the methodology and production of the data cited in Table VIII-3 has been added to the PDF reference, included with this submission.

   The discrepancy between the percent of maize acres treated with the herbicide glyphosate in Table VIII-3 in the petition and Table 2 in the USDA-NASS 2019b reference is due to reporting of a sub-set of glyphosate use data in the USDA-NASS 2019b summary report. Table 2 from the USDA-NASS 2019b only reports a single glyphosate formulation (isopropylamine salt) use in maize (34% acres treated) while the total maize acres treated with herbicide glyphosate irrespective of its formulations was approximately 76% as per the pesticide use data available at USDA-NASS survey and is summarized below. Table VIII-3 in the petition includes total glyphosate use in maize irrespective of its formulations.

Glyphosate formulations	**Percent of maize acres treated**
CHEMICAL, HERBICIDE: (GLYPHOSATE = Code: 417300)	12%
CHEMICAL, HERBICIDE: (GLYPHOSATE AMM. SALT = Code: 103604)	Data not available
CHEMICAL, HERBICIDE: (GLYPHOSATE DIM. SALT = Code: 103608)	4%
CHEMICAL, HERBICIDE: (GLYPHOSATE ISO. SALT = Code: 103601)	34%*
CHEMICAL, HERBICIDE: (GLYPHOSATE POT. SALT = Code: 103613)	26%
Total per USDA NASS Survey	**~76%**

*Published in USDA-NASS 2019b.

[1] Monsanto Company remains a legal entity holding registrations, permits and other approvals, but it is wholly owned by Bayer. We will let you know about any reorganization of legal entities, including transfer of authorizations/registrations from Monsanto and/or address updates. The names of entities holding registrations should not have any bearing on our approvals. These changes are for legal administrative reasons and have no influence on the quality of the end-use products, grain or seeds and they have no consequences for existing safety evaluations.

2. Typo has been corrected to chloroacetamide.
3. Buckhorn plantain has been used throughout.
4. Delye et al., 2003b has been added as a citation.
5. Hidayat and Preston 1997 PDF is provided with this submission.
6. "+MCPA" has been added to the recommendation for kochia control in wheat in Table K-1.

EXHIBIT F

Five-Herbicide Corn Tech

Bayer's Future Five-Way Herbicide-Tolerant Corn Under Review by USDA

4/28/2021 | 2:38 PM CDT



By Emily Unglesbee, DTN Staff Reporter
Connect with Emily:
@Emily_Unglesbee



Bayer is developing a corn technology that would tolerate in-season applications of five herbicides -- dicamba, 2,4-D, glufosinate, glyphosate and quizalofop. USDA is currently reviewing its potential environmental impact. (DTN photo by Greg Horstmeier)

ROCKVILLE, Md. (DTN) -- USDA is considering how to regulate Bayer's developmental five-way herbicide-tolerant corn trait, which will ultimately tolerate in-season applications of 2,4-D, dicamba, glufosinate, glyphosate and quizalofop (FOPS herbicides).

USDA's Animal and Plant Health Inspection Service (APHIS) received a petition from Monsanto (now a legal entity owned by Bayer) to deregulate the new GM corn trait, called MON 87429, last spring, so the company can use it for hybrid seed production. After reviewing comments from the public, the agency announced its next step this week: the launch of an environmental impact review for the new trait.

"In reviewing the public comments and information received during and after the comment period on Bayer's petition, APHIS concluded that an environmental impact statement is necessary to assess the potential impacts to the environment from deregulating MON 87429 maize," the agency said in its news release.

The new five-way trait would replace Bayer's development of XtendFlex corn, a three-way trait that tolerates dicamba, glufosinate and glyphosate and was deregulated by USDA in 2016. The company is still waiting on EPA to approve use of current dicamba herbicides on XtendFlex corn, so it isn't yet commercialized. In the interim, Bayer has been working on this new, five-way traited corn, which it calls HT4, and is re-focusing much of its research efforts there.

"Considering the progress of these two events through the global regulatory review process and other factors, we have decided to pause work on hybrid development for our third generation herbicide-tolerant corn (HT3) product [XtendFlex corn] and are focusing our resources on our fourth generation herbicide-tolerant corn (HT4) product," Bayer said in an emailed statement to DTN. "We are excited about our HT4 corn product that, once commercialized, will offer the most complete herbicide-tolerant package for corn in the marketplace."

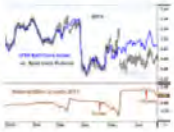
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"Pending regulatory approvals and other factors, we anticipate a commercial launch of our HT4 corn product mid-to-late this decade," the statement added.

In its petition for deregulation, Bayer told EPA the new five-way traited corn hybrids would help fight weed resistance. Glyphosate resistance is now widespread in the U.S., and scientists in some Southern states have also already detected pigweed populations resistant to dicamba, glufosinate and possibly 2,4-D. (See more from DTN here: https://www.dtnpf.com/... and here: https://www.dtnpf.com/....)

The new corn stack would allow postemergence use of all five herbicides, but Bayer's petition claims it won't change corn herbicide use, largely because this particular petition for deregulation is only for hybrid seed production. As a result, the company didn't have to create estimates for how many acres might be planted to commercialized corn plants sporting MON 87429 yet.

Bayer also stated that the new, five-way stack will not require different label rates or spray windows in corn from those already established for current glyphosate, glufosinate, quizalofop and 2,4-D herbicides.

Dicamba is the exception to that. In its petition for deregulation, Bayer states that the five-way traited corn would use the same application timing (through the V8 growth stage) and rates of dicamba (up to 2 pounds of dicamba acid equivalent per year) as that XtendFlex corn trait, which USDA has already reviewed and deregulated. However, those expanded spray windows and rates for dicamba use in corn have not yet been approved by EPA, which governs pesticide use, and are not in use on the landscape yet.

As a result, Bayer acknowledges that dicamba-tolerant technology in corn could expand dicamba use in corn. Specifically, in regulatory documents for XtendFlex corn, Bayer estimated that once fully commercialized, dicamba-tolerant corn could eventually be planted on 89% of all U.S. corn acres, with up to 36% of all corn acres receiving a dicamba application.

However, the company has underestimated use of its dicamba-tolerant cotton and soybean acres to USDA in the past. Environmental activist groups such as the Center for Food Safety argue dicamba use will be higher than suggested for dicamba-tolerant corn. These groups are lobbying for USDA to reject the petition, which received more than 4,100 public comments when it was posted last May.

"(Bayer/)Monsanto's petition to approve a new GM corn variety resistant to five herbicides, including dicamba, is beyond the pale," Bill Freese, science director for the Center for Food Safety said in an email to DTN. "The company's dicamba-resistant soybeans and cotton have already had devastating impacts on American agriculture, with rampant dicamba drift causing unprecedented injury to millions of acres of soybean, orchards, vegetable farms and trees throughout rural America, and this new GM corn would make things immeasurably worse."

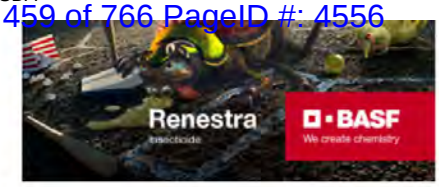
See the petition for deregulation of Bayer's five-way herbicide-tolerant corn trait, MON 87429 and USDA's most recent actions on it here: https://www.regulations.gov/....

See more on Bayer's XtendFlex corn here: https://www.dtnpf.com/...

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EXHIBIT G

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US011149283B2

(12) **United States Patent**
Wright et al.

(10) **Patent No.: US 11,149,283 B2**
(45) **Date of Patent: *Oct. 19, 2021**

(54) **HERBICIDE RESISTANCE GENES**

(71) Applicant: **DOW AGROSCIENCES LLC**, Indianapolis, IN (US)

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(73) Assignee: **Dow Agrosciences LLC**, Indianapolis, IN (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **17/175,966**

(22) Filed: **Feb. 15, 2021**

(65) **Prior Publication Data**

US 2021/0254090 A1 Aug. 19, 2021

Related U.S. Application Data

(63) Continuation of application No. 17/143,824, filed on Jan. 7, 2021, which is a continuation of application No. 15/288,406, filed on Oct. 7, 2016, now Pat. No. 10,947,555, which is a continuation of application No. 14/820,893, filed on Aug. 7, 2015, now Pat. No. 10,174,337, which is a continuation of application No. 12/951,813, filed on Nov. 22, 2010, now Pat. No. 9,127,289, which is a continuation of application No. 11/587,893, filed as application No. PCT/US2005/014737 on May 2, 2005, now Pat. No. 7,838,733.

(60) Provisional application No. 60/567,052, filed on Apr. 30, 2004.

(51) **Int. Cl.**
C12N 15/82 (2006.01)
C12N 9/02 (2006.01)

(52) **U.S. Cl.**
CPC ***C12N 15/8274*** (2013.01); ***C12N 9/0069*** (2013.01); ***C12N 9/0071*** (2013.01); ***C12N 15/8209*** (2013.01)

(58) **Field of Classification Search**
CPC C12N 15/8274
See application file for complete search history.

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(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.

29 Claims, 24 Drawing Sheets

Specification includes a Sequence Listing.

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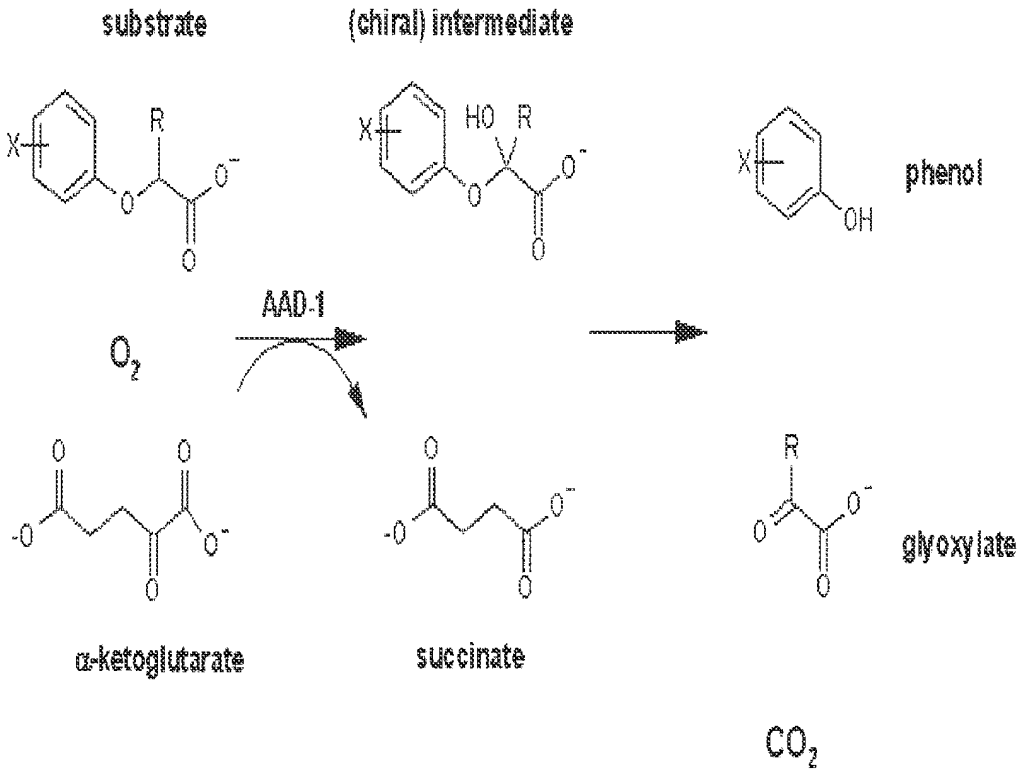
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Final Decision in Opposition filed against EP 10012199.5, dated Feb. 9, 2016.

* cited by examiner



- Addition of O_2 is *stereospecific*
- Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

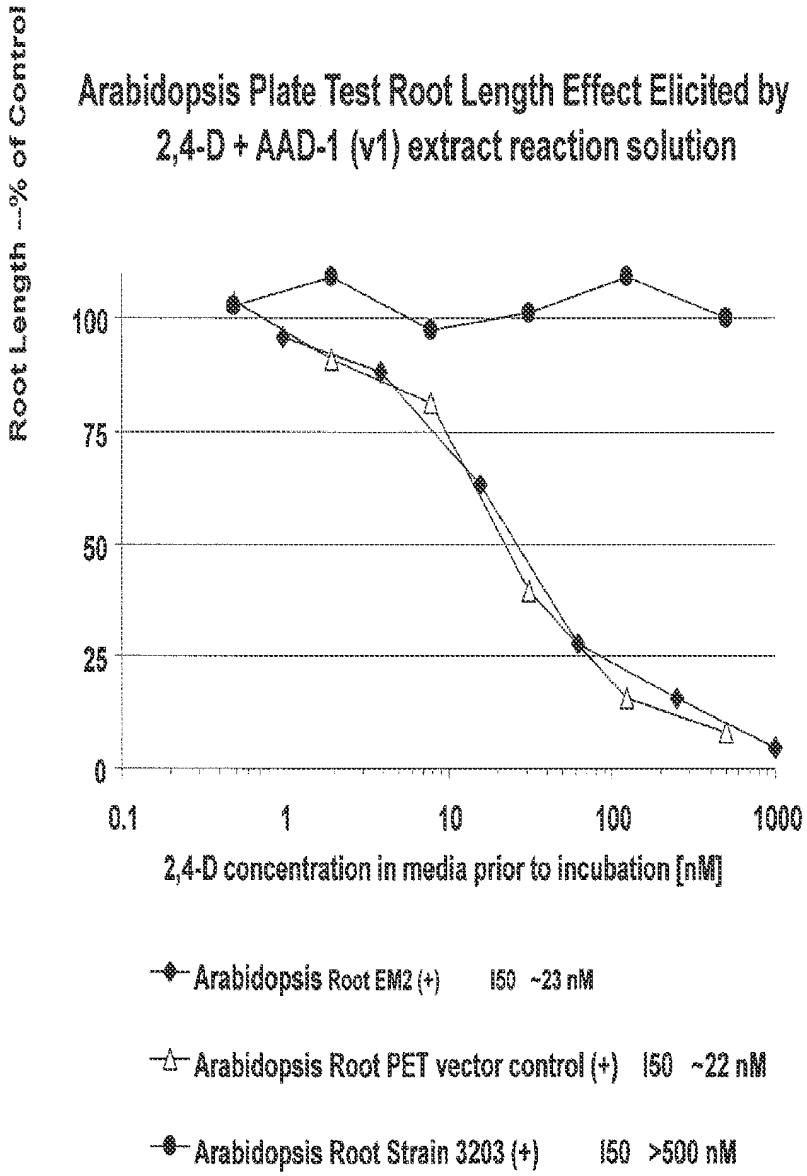

Arabidopsis Plate Test Root Length Effect Elicited by
2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

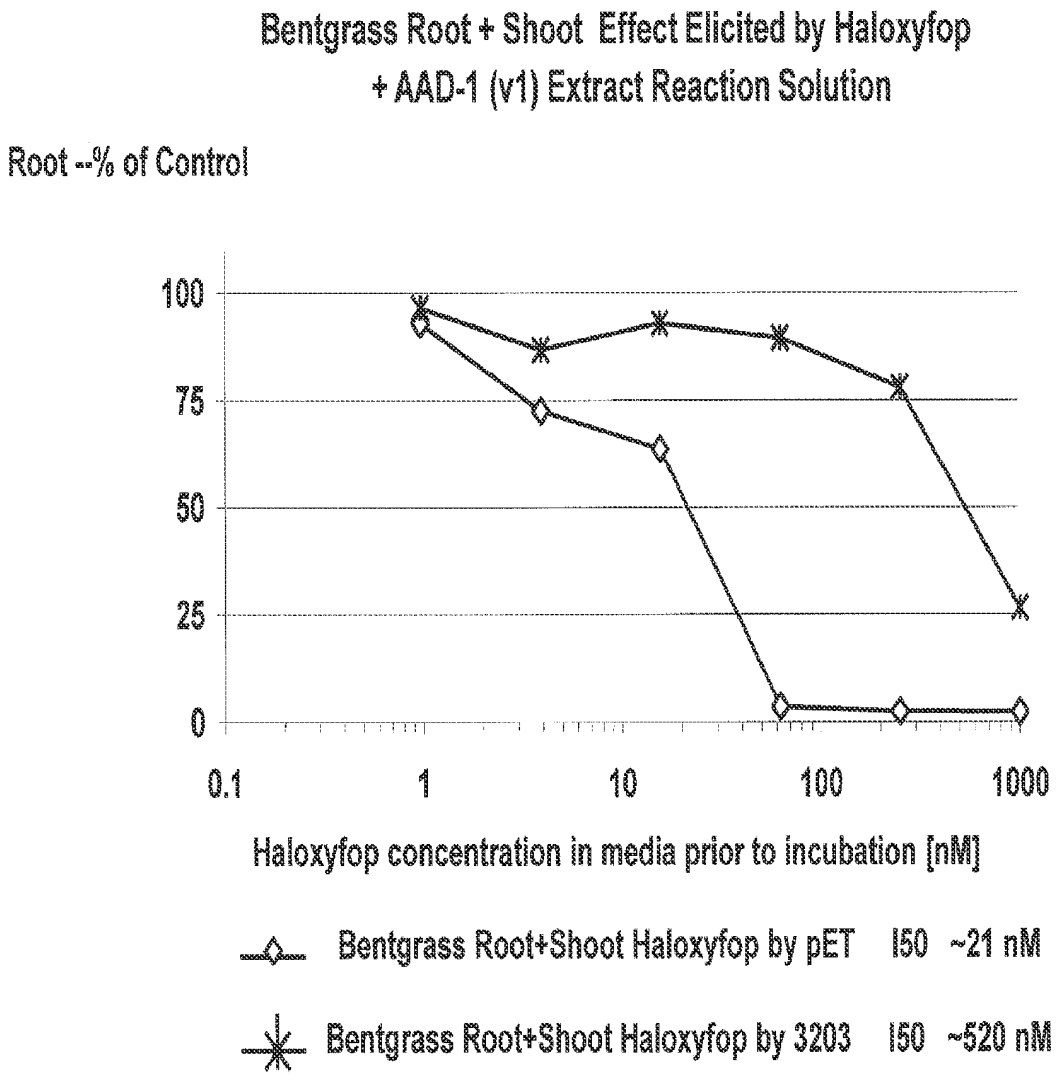

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

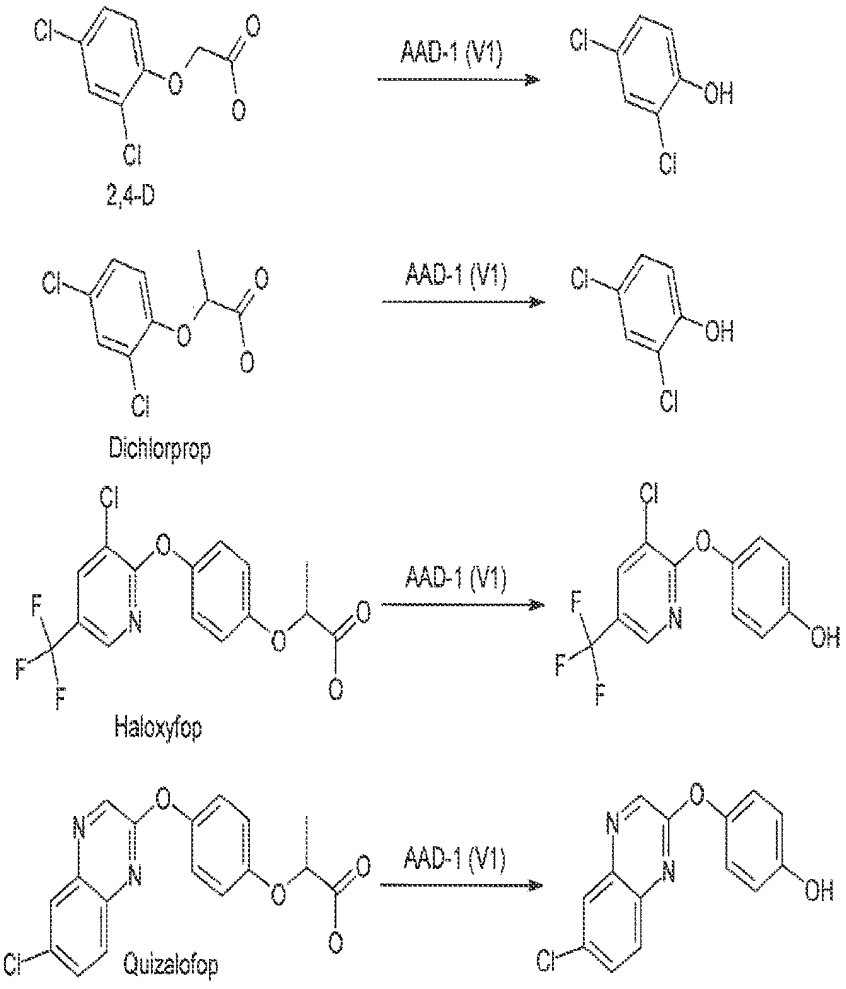

AAD-1 (V1)
2,4-D
AAD-1 (V1)
Dichlorprop
AAD-1 (V1)
Haloxyfop
AAD-1 (V1)
Quizalofop

Fig. 4

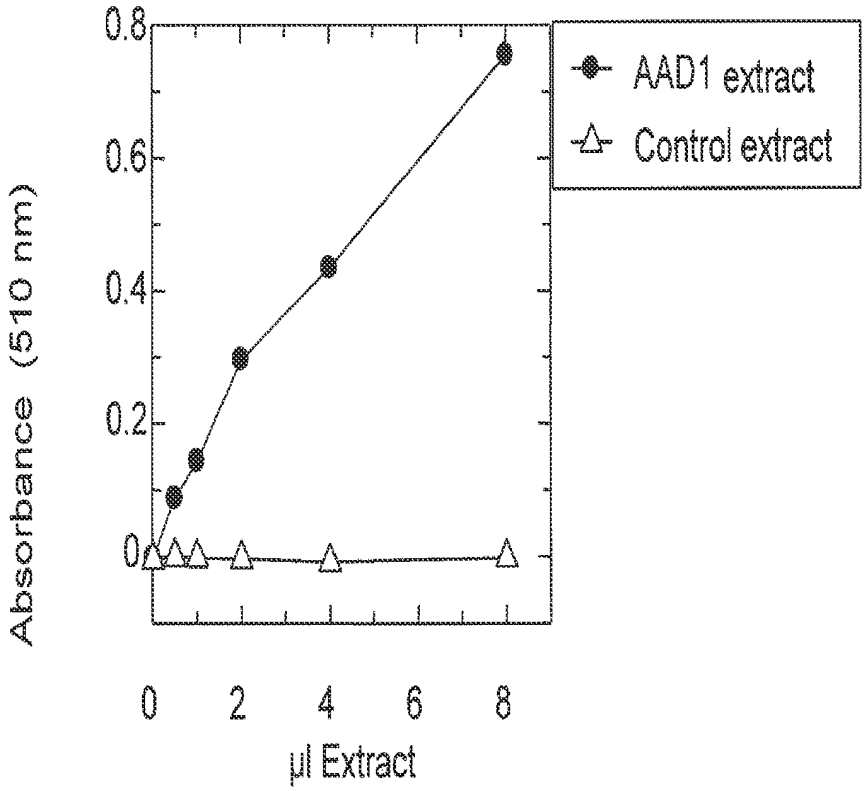

0.8
0.6
0.4
0.2
0
Absorbance (510 nm)
0
2
4
6
8
µl Extract
AAD1 extract
Control extract

Fig. 5

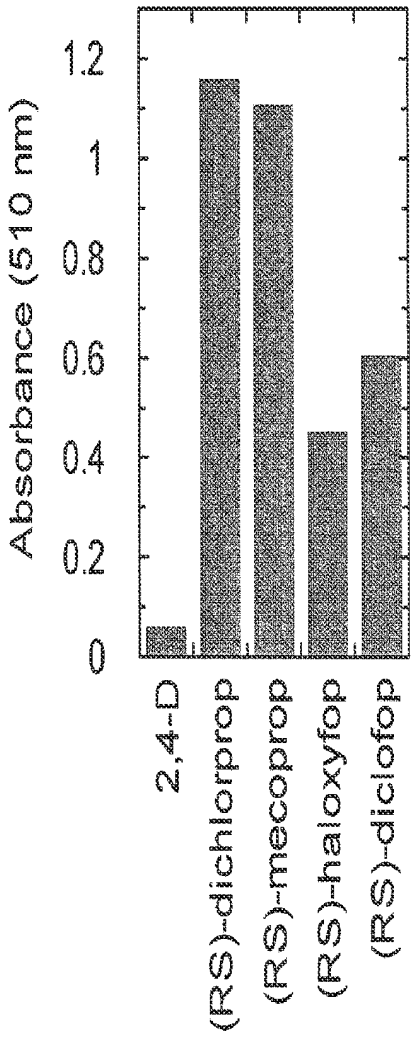

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
2,4-D
(RS)-dichlorprop
(RS)-mecoprop
(RS)-haloxyfop
(RS)-diclofop

Fig. 6A

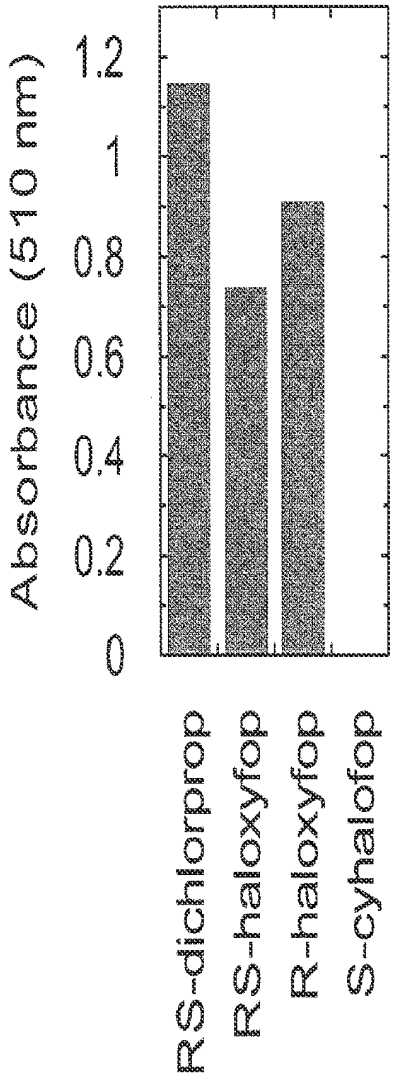

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

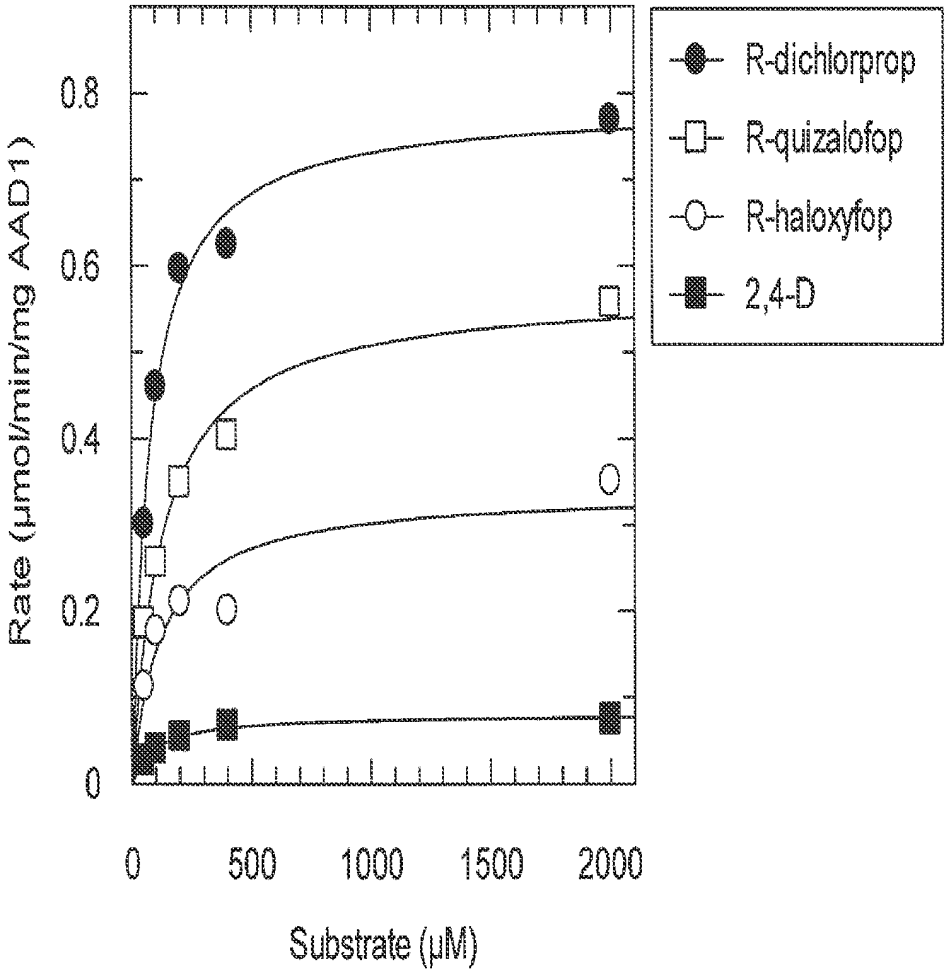

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

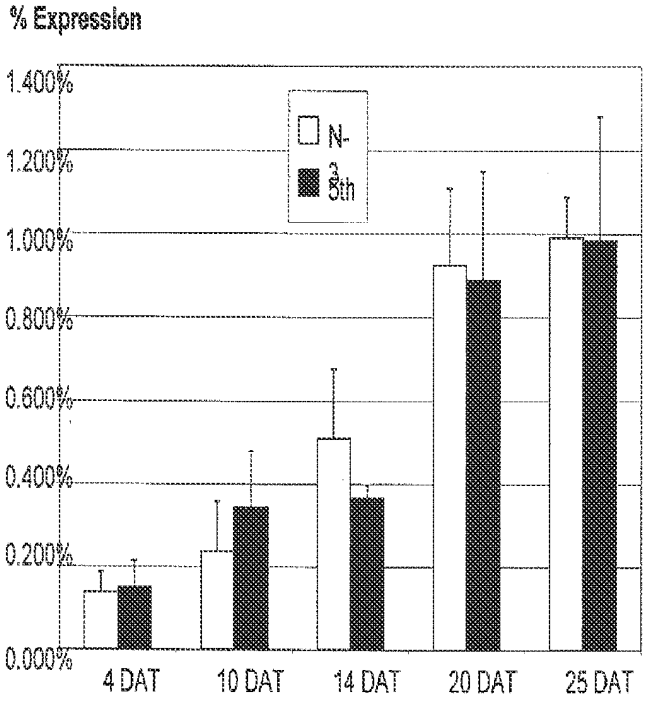

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

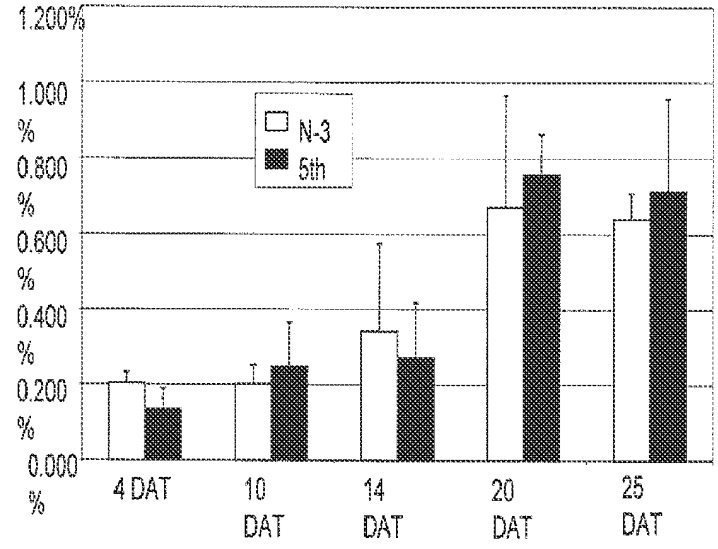

% Expression
1.200%
1.000 %
0.800 %
0.600 %
0.400 %
0.200 %
0.000 %
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

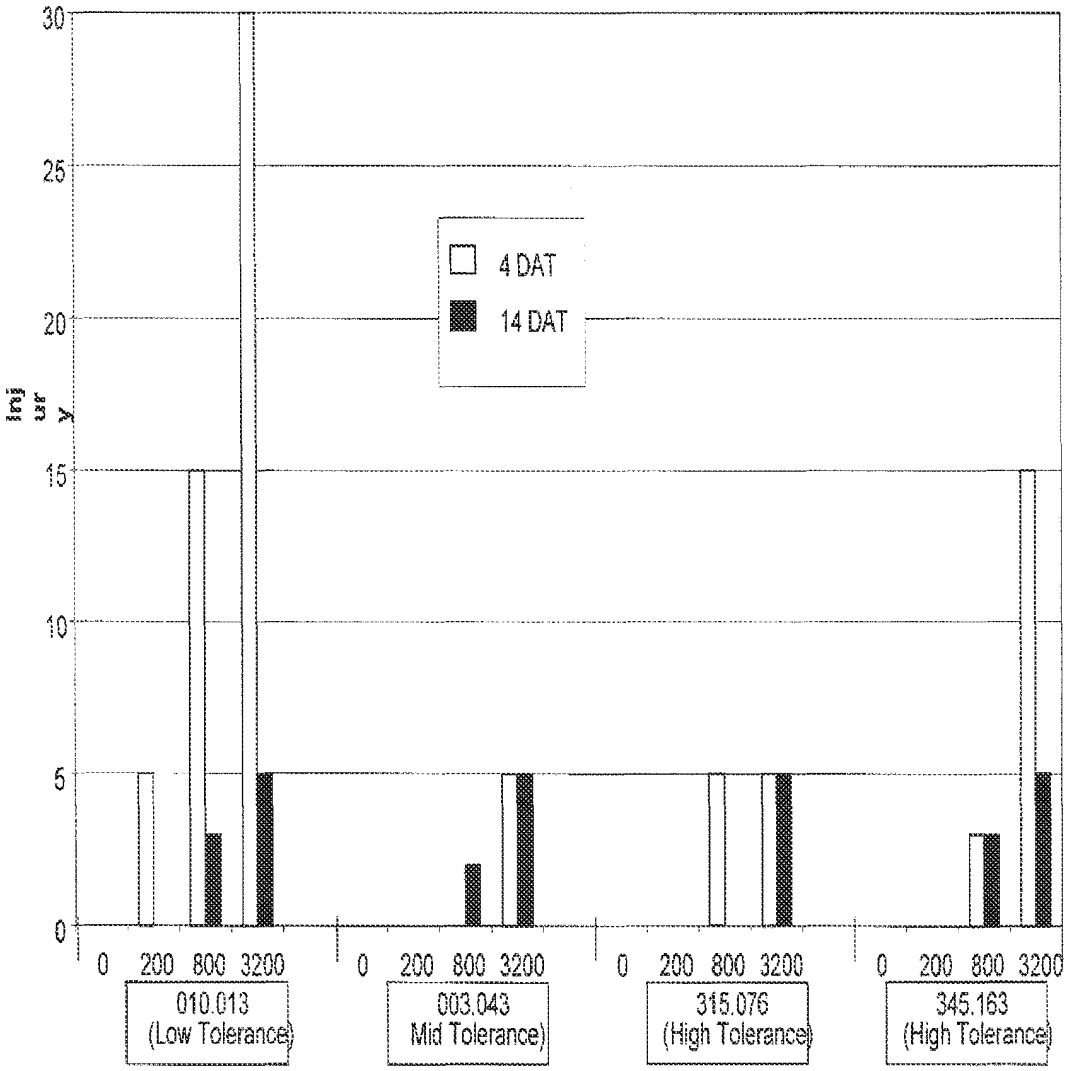

30
25
20
15
10
5
0
4 DAT
14 DAT
0 200 800 3200
010.013
(Low Tolerance)
0 200 800 3200
003.043
Mid Tolerance)
0 200 800 3200
315.076
(High Tolerance)
0 200 800 3200
345.163
(High Tolerance)

Fig. 9A

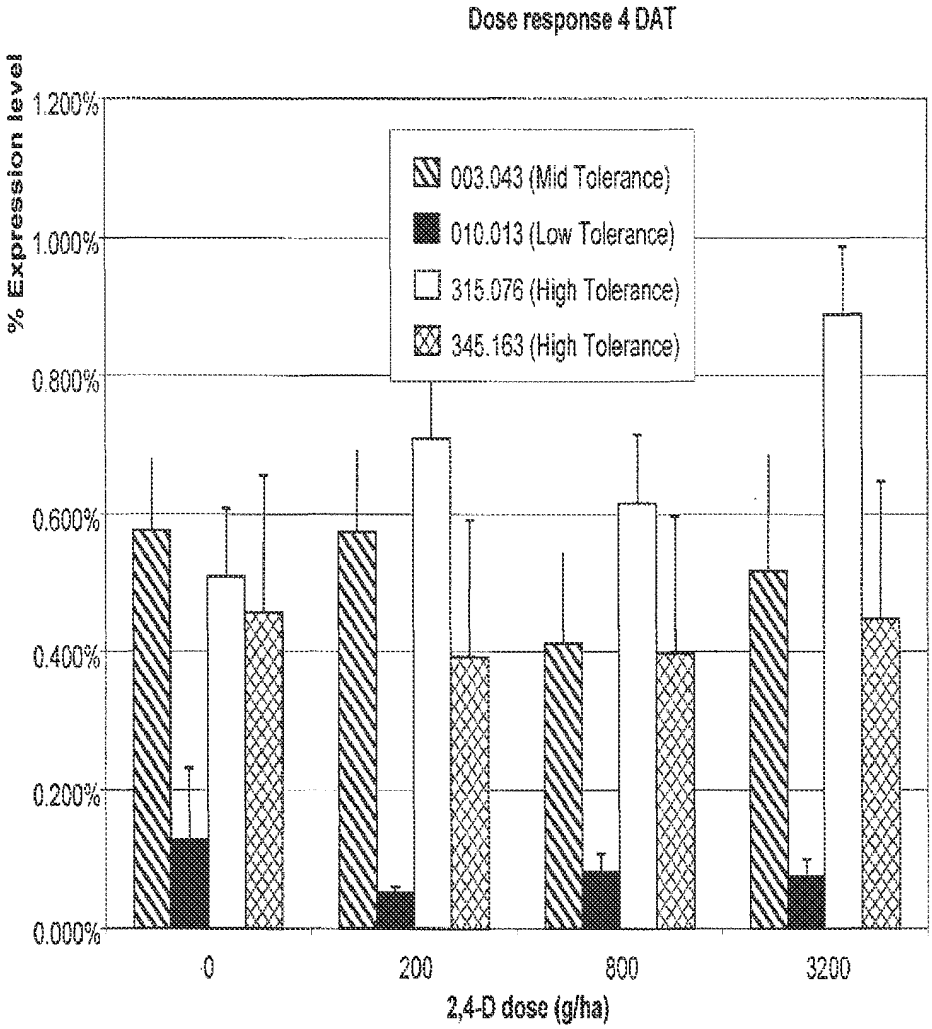

Dose response 4 DAT
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

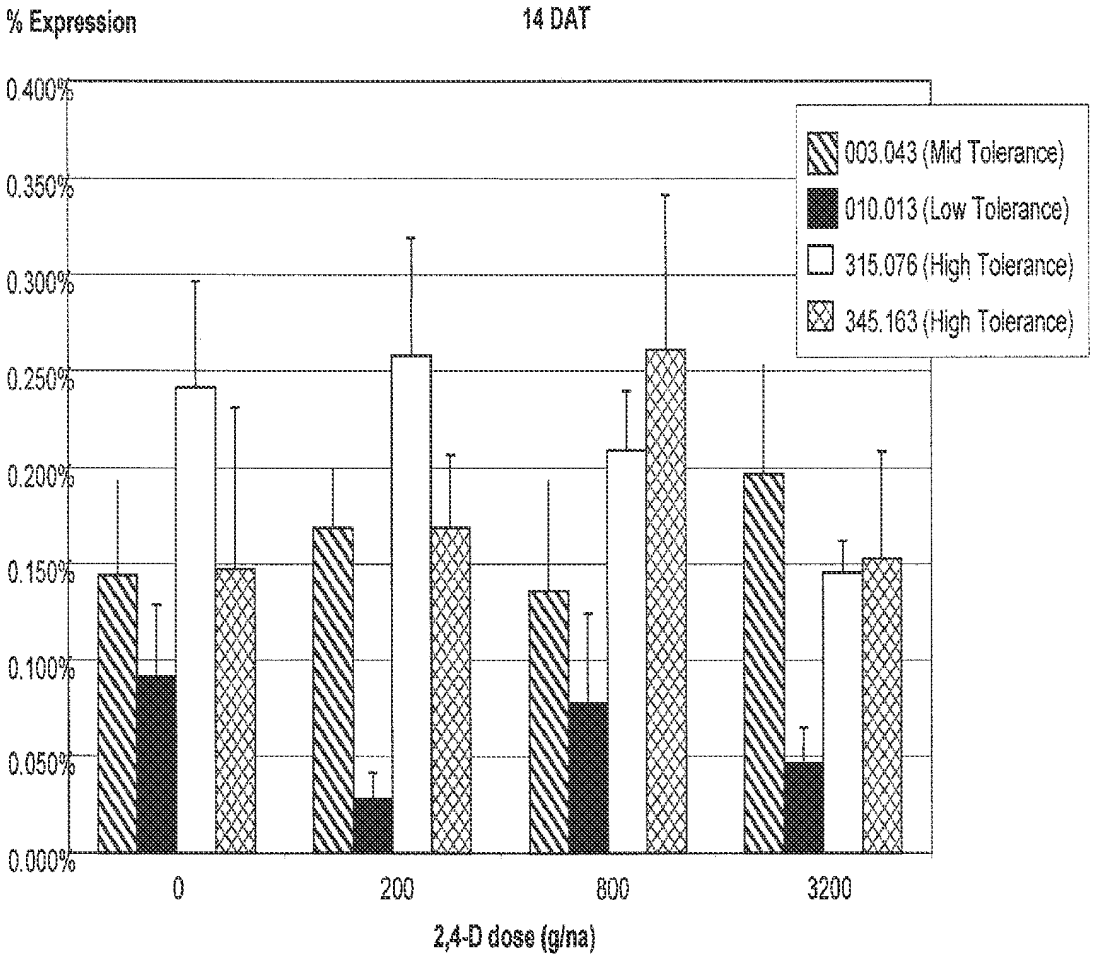

% Expression
14 DAT
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

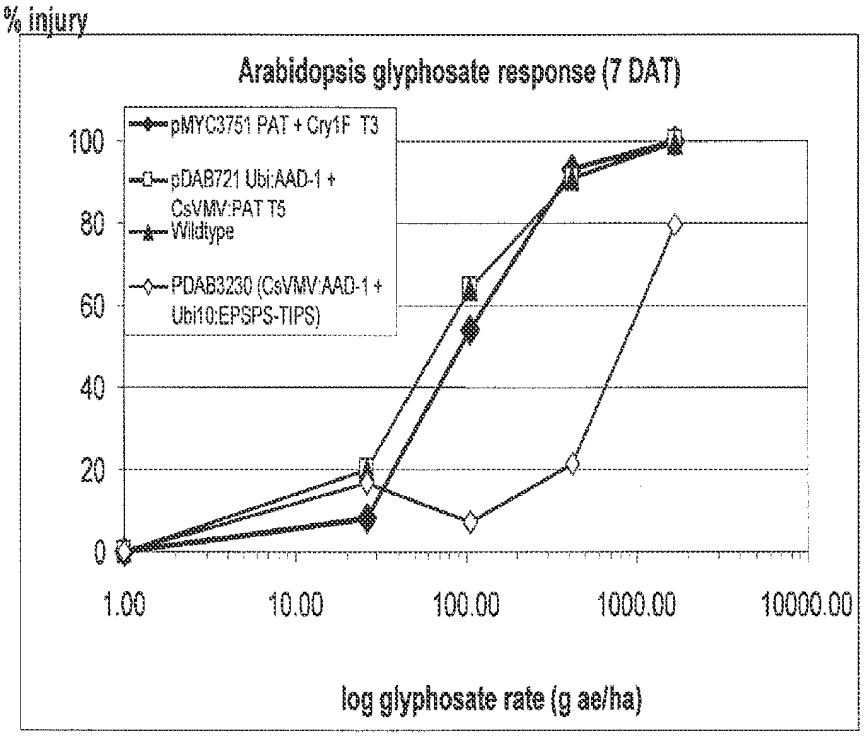

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

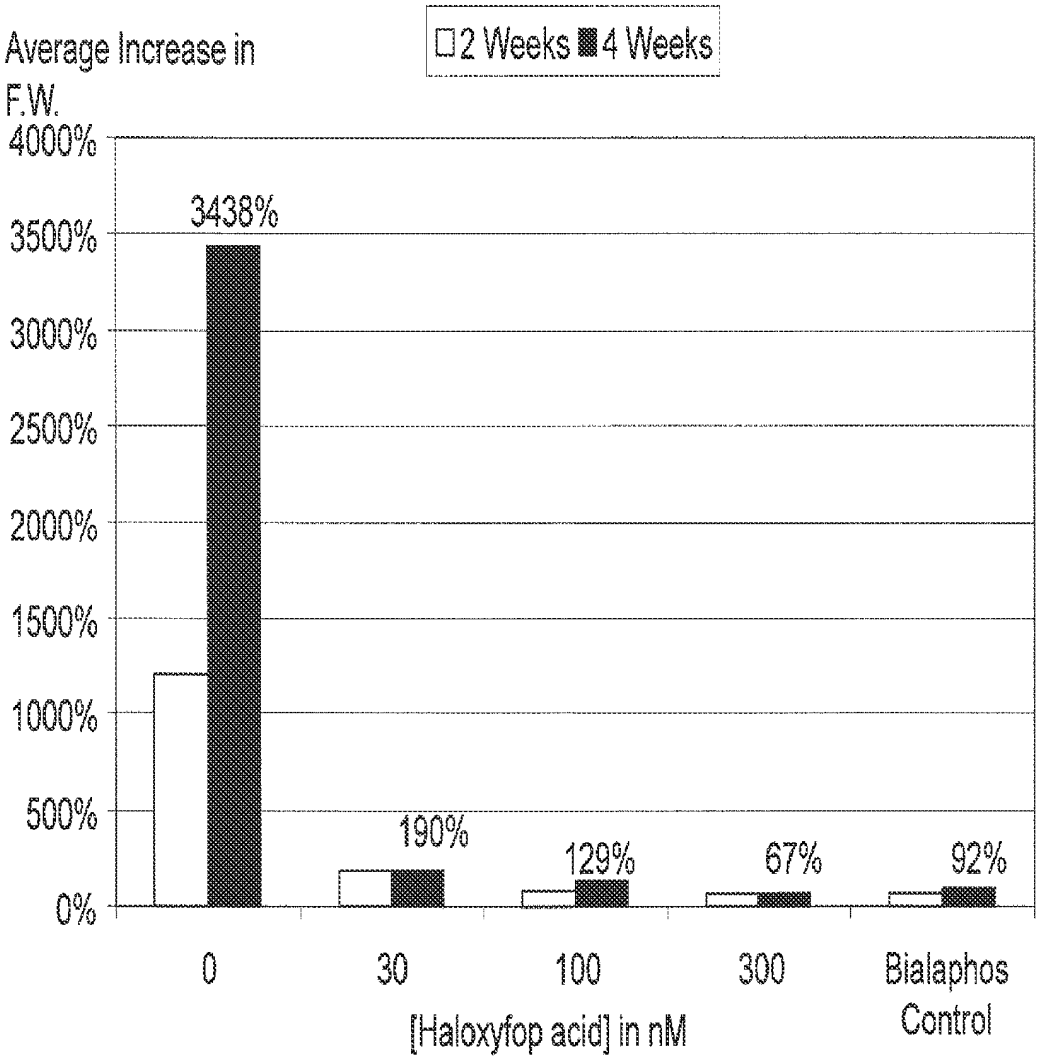

Average Increase in F.W.
2 Weeks
4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

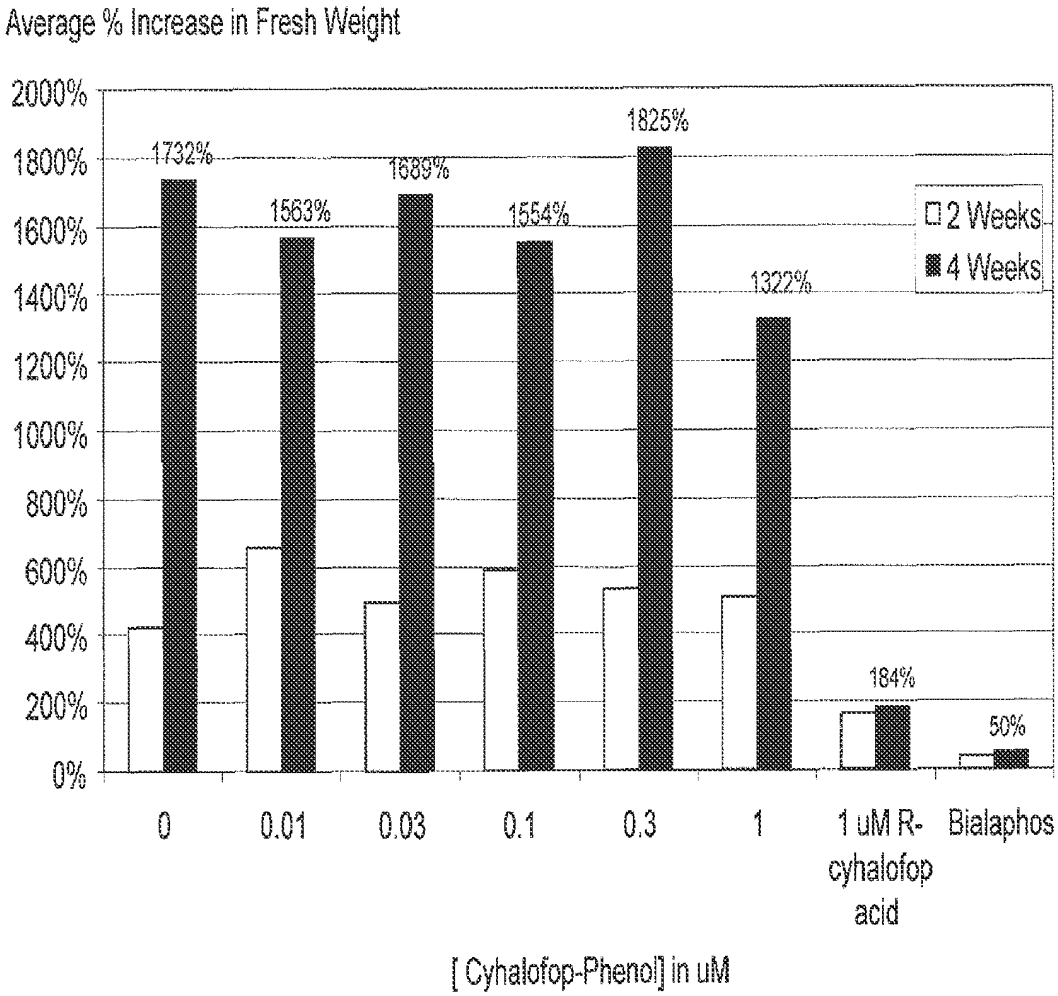

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
2 Weeks
4 Weeks
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

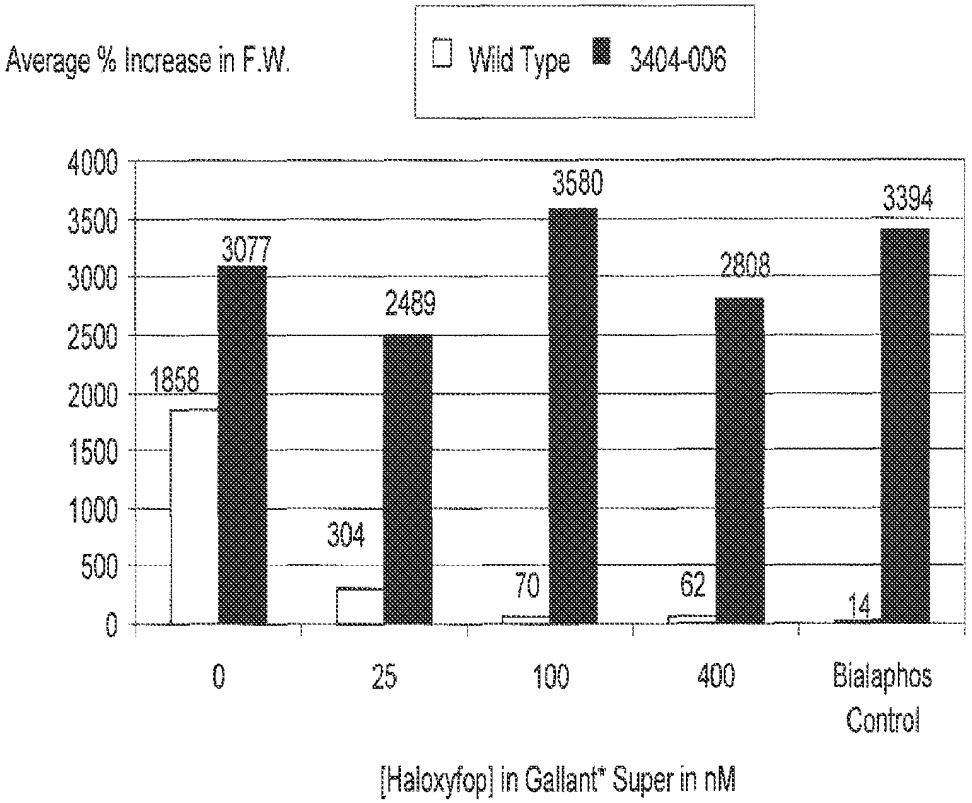

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
1858
3077
304
2489
70
3580
62
2808
14
3394
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant® Super in nM

Fig. 13

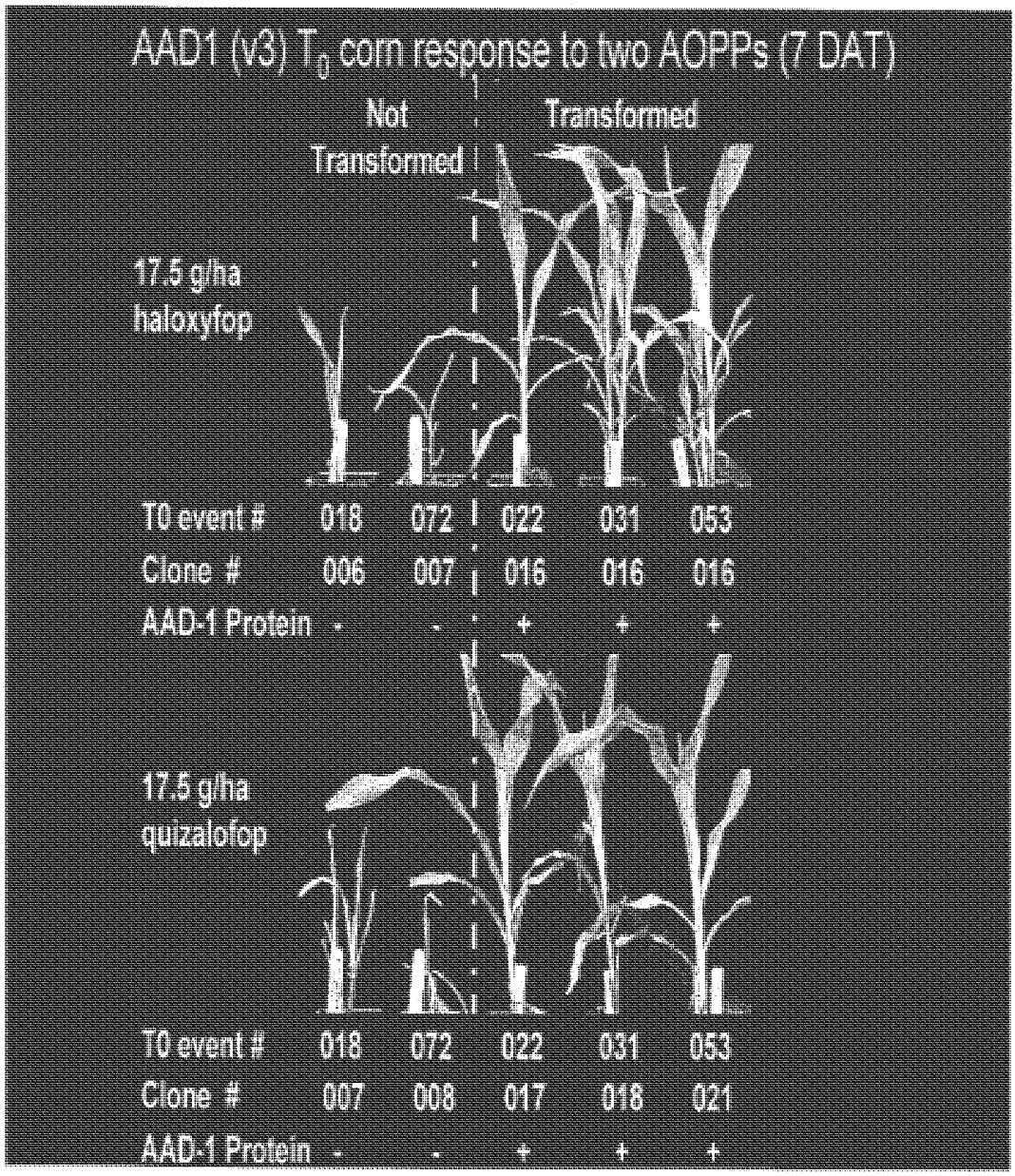

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

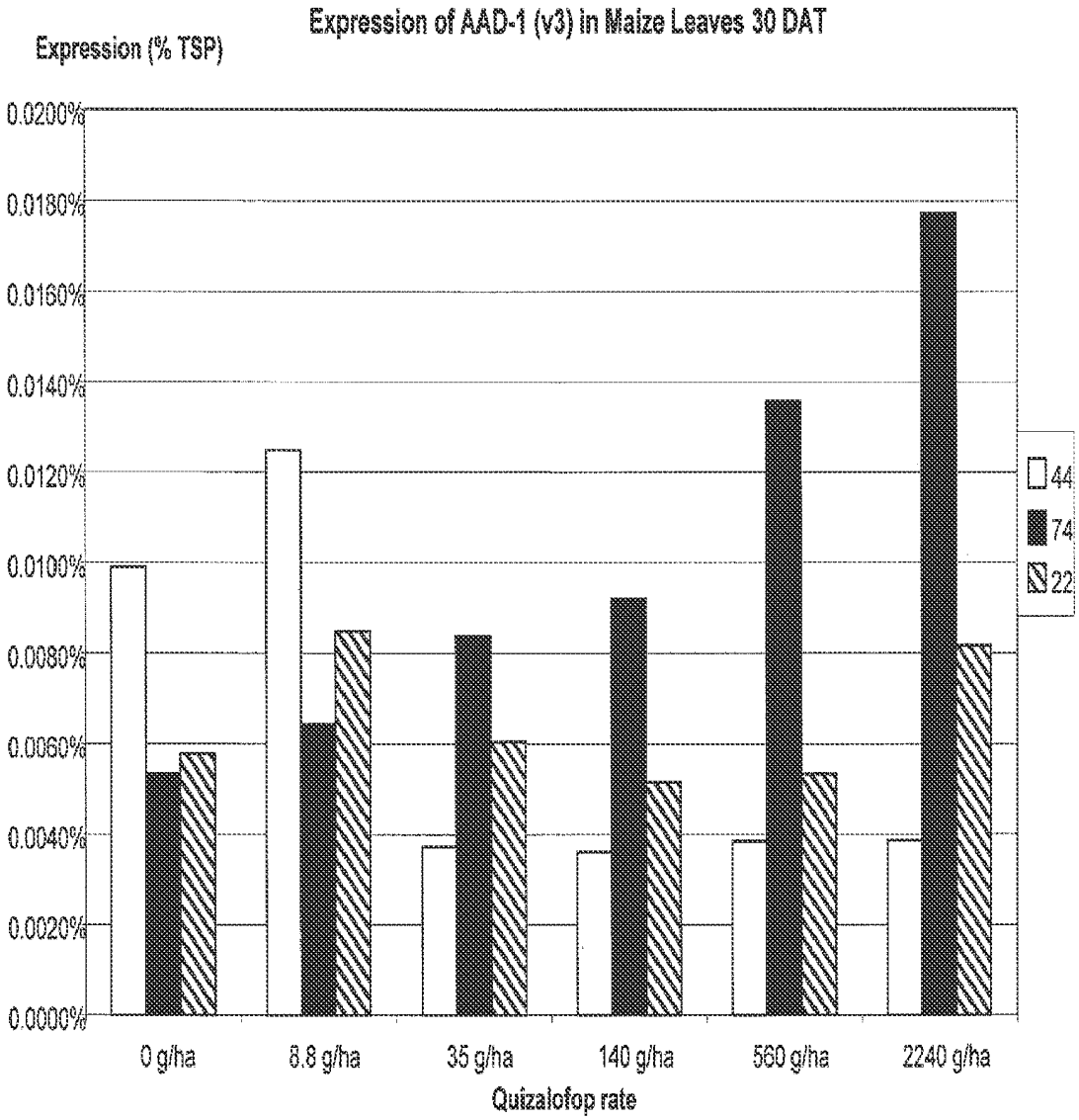

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate
44
74
22

Fig. 15

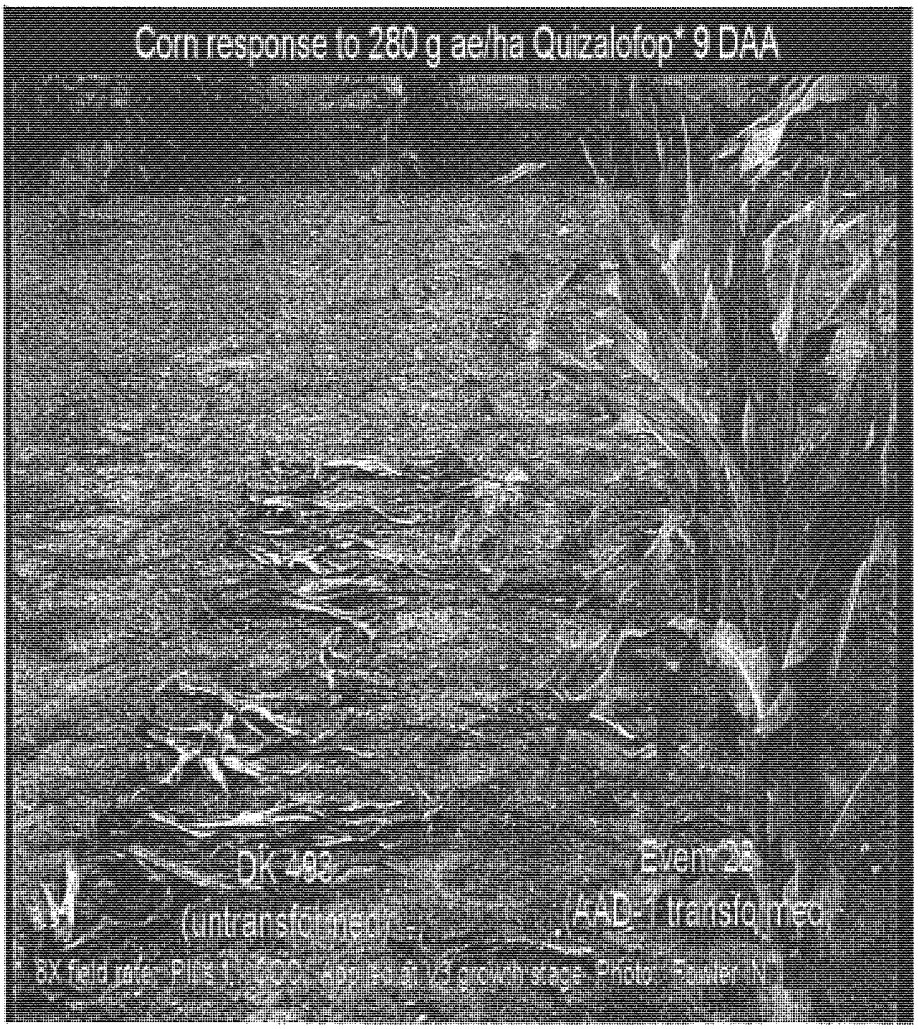

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 480
(untransformed)
Event 2
AAD-1 (transformed)

Fig. 16

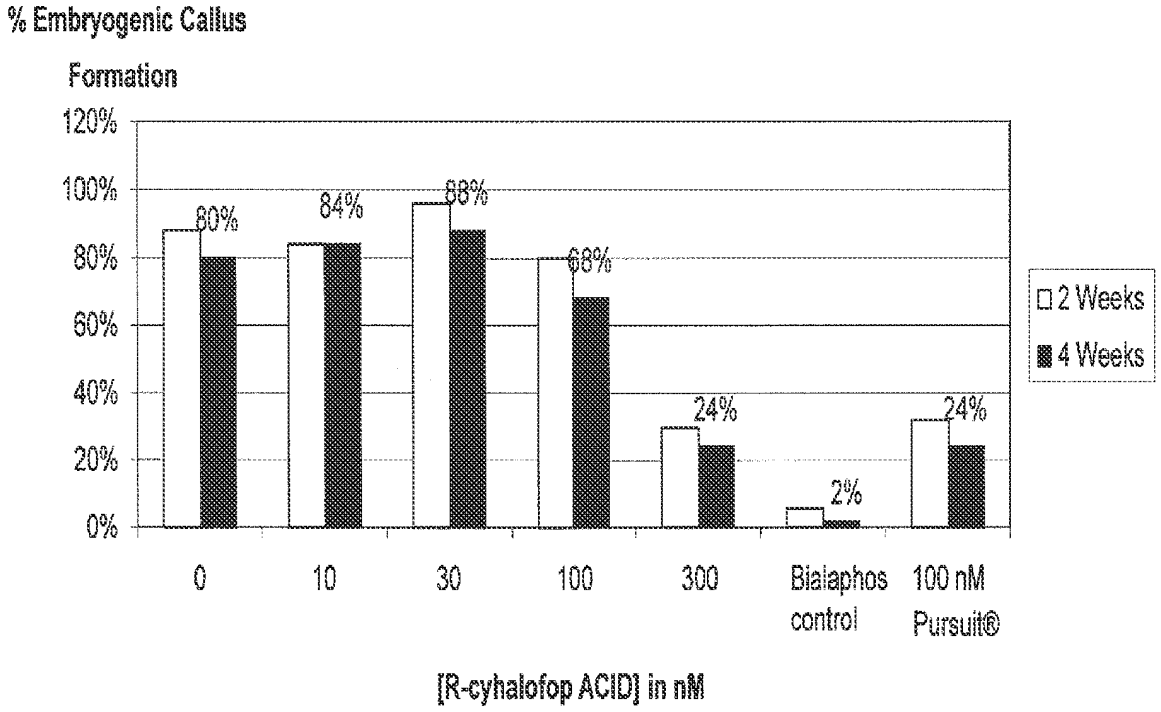
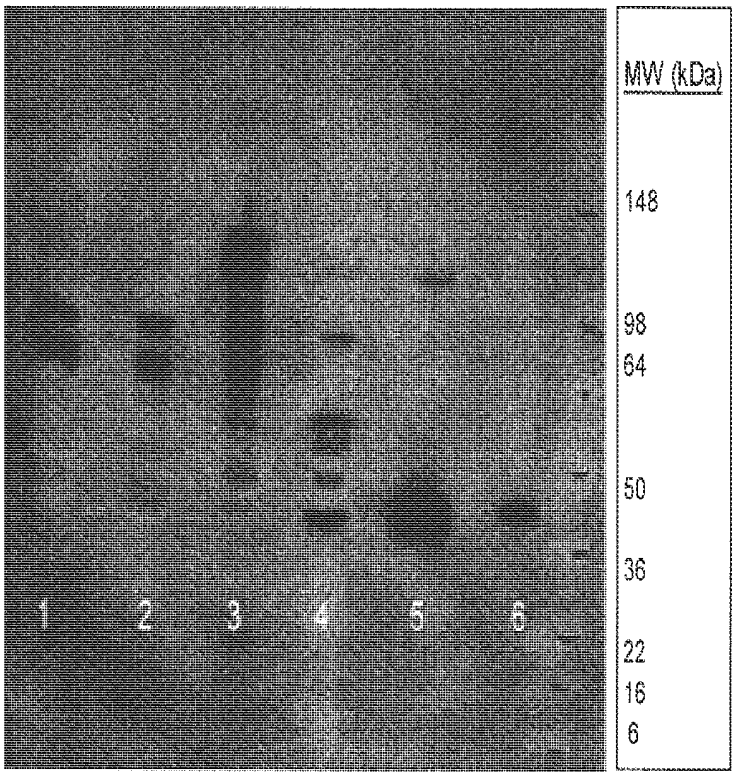

% Embryogenic Callus
Formation
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
2 Weeks
4 Weeks
[R-cyhalofop ACID] in nM

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

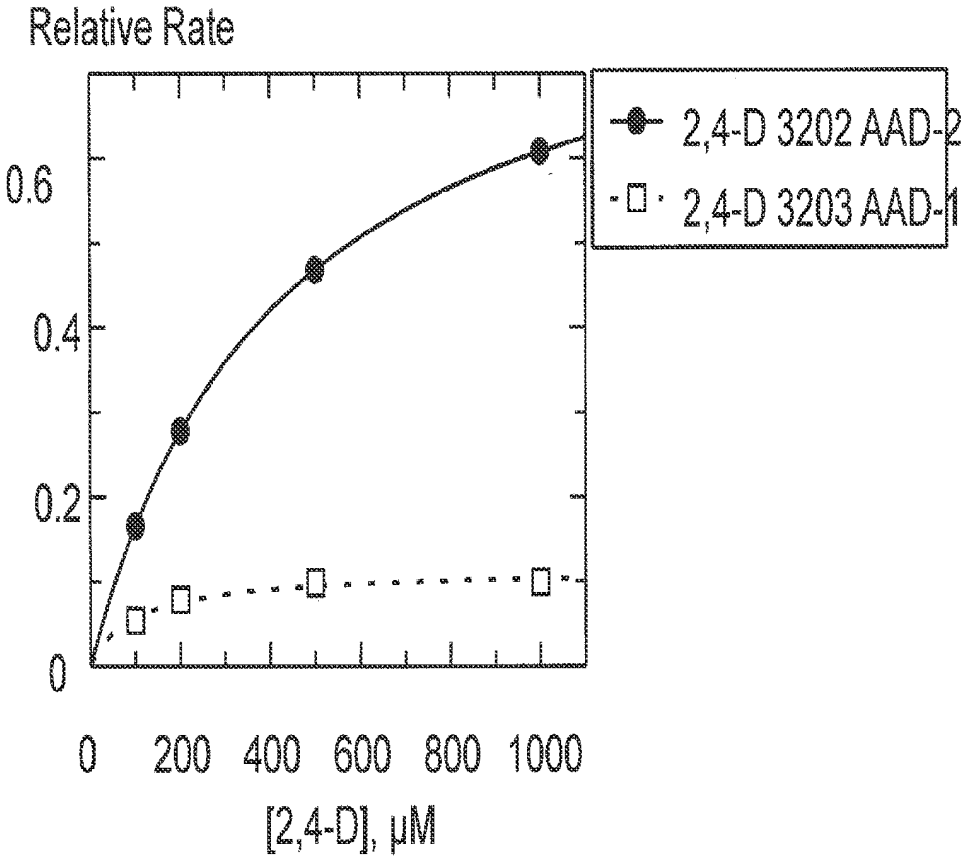

Relative Rate
0.6
0.4
0.2
0
0
200
400
600
800
1000
[2,4-D], µM
2,4-D 3202 AAD-2
2,4-D 3203 AAD-1

Fig. 19

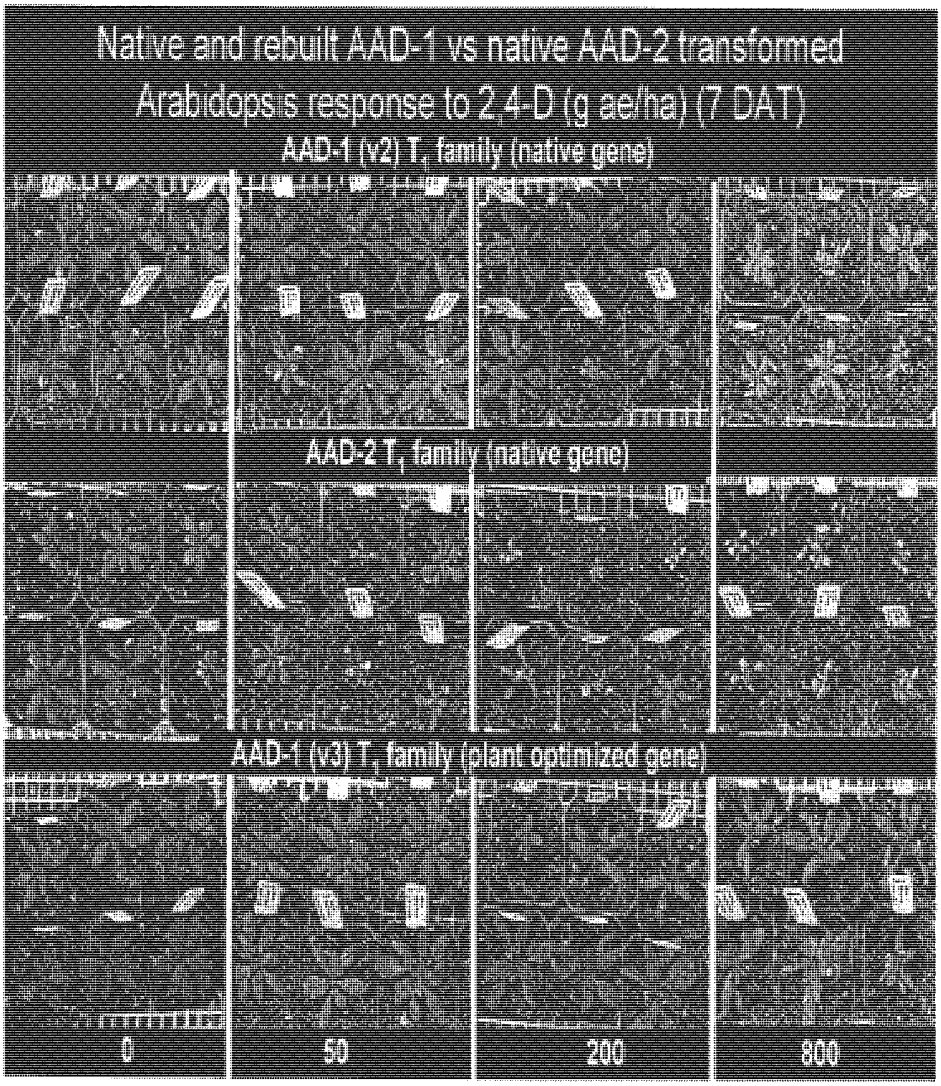

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

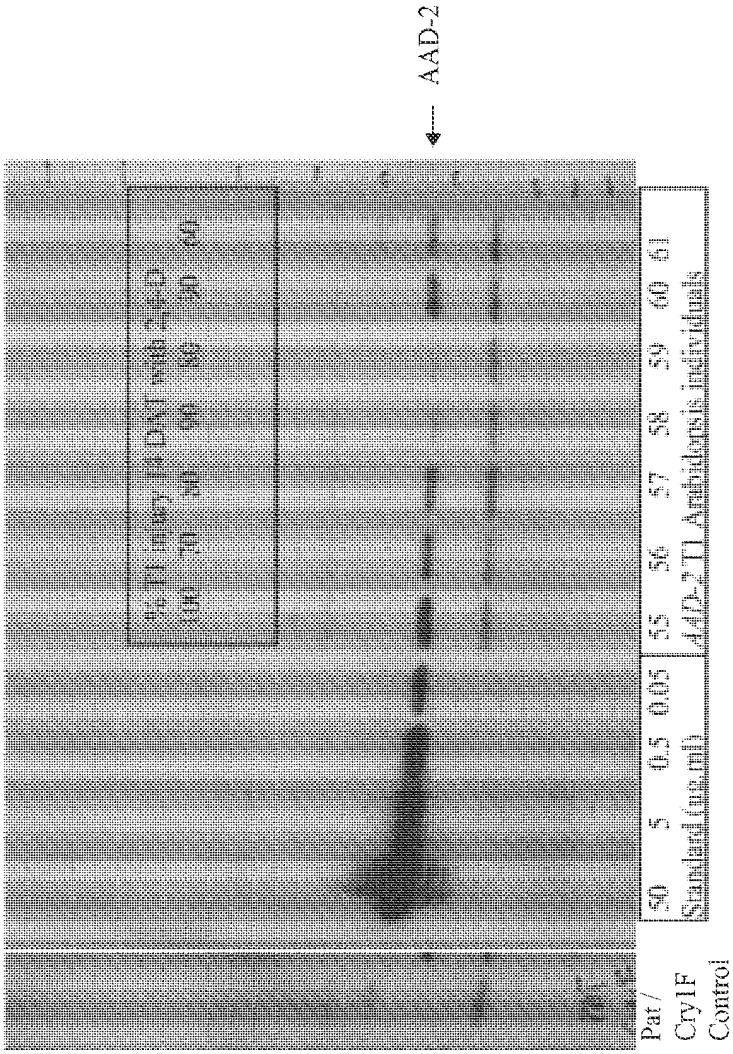

AAD-2
Pat/Cry1F Control
50 5 0.5 0.05 Standard (ug/mL)
55 56 57 58 59 60 61 AAD-2 T1 Antibiotic individuals

Fig. 21

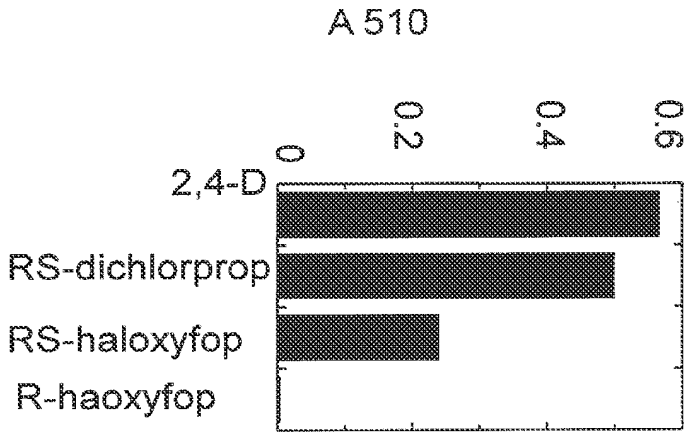


FIG. 22

HERBICIDE RESISTANCE GENES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation application of U.S. Ser. No. 17/143,824, filed Jan. 7, 2021, which is a continuation application of U.S. Ser. No. 15/288,406, filed Oct. 7, 2016, now patented as U.S. Pat. No. 10,947,555, which is a continuation application of U.S. Ser. No. 14/820,893, filed Aug. 7, 2015, now patented as U.S. Pat. No. 10,174,337, which is a continuation application of U.S. Ser. No. 12/951,813, filed Nov. 22, 2010, now patented as U.S. Pat. No. 9,127,289, which is a continuation application of U.S. Ser. No. 11/587,893, filed May 22, 2008, now patented as U.S. Pat. No. 7,838,733, which is a national stage entry of PCT/US2005/014737, filed May 2, 2005, which claims the benefit of U.S. Provisional Application Ser. No. 60/567,052, filed Apr. 30, 2004, which are hereby incorporated by reference in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

INCORPORATION OF SEQUENCE LISTING

Incorporated by reference in its entirety is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herein and identified as follows: One 42 KB ACII (Text) file named "334171_ST25" created on Apr. 7, 2021.

BACKGROUND OF THE INVENTION

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.

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.

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.

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.

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.

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).

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.

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).

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.

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. Pat. 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. Pat. No. 5,608,147).

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.

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).

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.

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

BRIEF SUMMARY OF THE INVENTION

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.

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).

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.

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.

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

FIG. **1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

FIG. **2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

FIG. **3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

FIG. **4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

FIG. **5** shows 2,4-dichlorophenol production by recombinant AAD-1.

FIGS. **6**A and **6**B show phenol production by recombinant AAD-1 from various herbicide substrates.

FIG. **7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

FIGS. **8**A and **8**B 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.

FIGS. **9**A, **9**B, and **9**C 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.

FIG. **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.

FIG. **11** shows dose response of callused maize suspensions to R-haloxyfop.

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

FIG. **13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

FIG. **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.

FIG. **15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

FIG. **16** shows AAD-1 (v3)-transformed corn tolerant to 8× field rates of quizalofop (Assure II) under field conditions.

FIG. **17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

FIG. **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.

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

FIG. **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.

FIG. **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.

FIG. **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

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

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

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

SEQ ID NO:4 is the nucleic acid sequence of the native AAD-1 gene with internal NotI restriction site removed. This gene is designated AAD-1 (v2). DNA sequencing confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

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.

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).

SEQ ID NO:8 is another PCR primer ("BstEII/Del NotI") that was used with the "3' SacI" primer.

SEQ ID NO:9 is the native amino acid sequence encoded by the AAD-1 (v1) gene from *Sphingobium herbicidovorans*.

SEQ ID NO:10 is the amino acid sequence encoded by the AAD-1 (v2) DNA sequence of SEQ ID NO:4.

SEQ ID NO:11 is the amino acid sequence encoded by the AAD-1 (v3) plant-optimized DNA sequence of SEQ ID NO:5.

SEQ ID NO:12 is the DNA sequence of the native AAD-2 (v1) gene.

SEQ ID NO:13 is the amino acid sequence of the AAD-2 (v1) protein.

SEQ ID NO:14 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:15 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:16 is the M13 forward primer.

SEQ ID NO:17 is the M13 reverse primer.

SEQ ID NO:18 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:19 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:20 is the native soybean EPSPS protein.

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).

SEQ ID NO:22 is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

SEQ ID NO:23 is primer Pat 5-3.

SEQ ID NO:24 is primer Pat 3-3.

SEQ ID NO:25 is forward primer AAD-1 PTU.

SEQ ID NO:26 is reverse primer AAD-1 PTU.

SEQ ID NO:27 is the forward primer for the Coding Region PCR AAD-1.

SEQ ID NO:28 is the reverse primer for the Coding Region PCR AAD-1.

SEQ ID NO:29 is the AAD-2 (v2) nucleotide (plant optimized).

SEQ ID NO:30 is the translated AAD-2 (v2) protein sequence.

SEQ ID NO:31 is the Southern fragment PCR AAD-1 forward primer.

SEQ ID NO:32 is the Southern fragment PCR AAD-1 reverse primer.

DETAILED DESCRIPTION OF THE INVENTION

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.

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

TABLE 1

Commercially available phenoxy auxins.

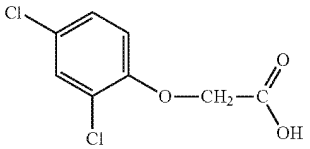
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	

TABLE 1-continued

Commercially available phenoxy auxins.

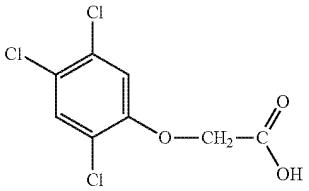
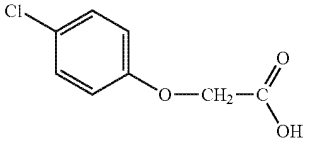
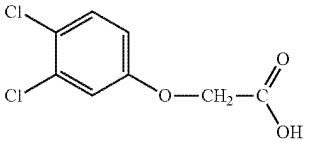
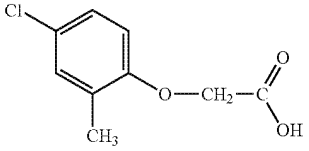
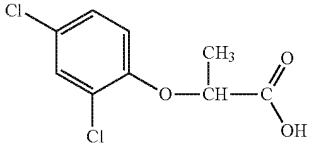
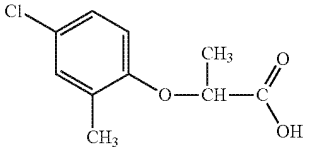
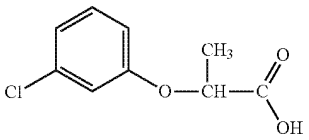
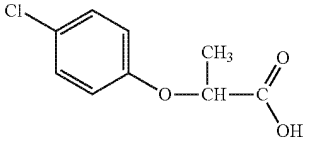
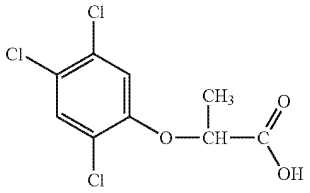
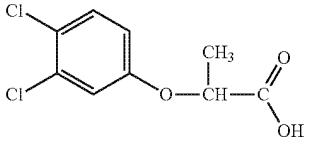
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4,5-T	93-76-5	25-4000	25-4000	
4-CPA	122-88-3	25-4000	25-4000	
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Dichlorprop	120-36-5	25-12000	100-2240	
Mecoprop	7085-19-0	25-4000	250-3360	
Cloprop	101-10-0	25-4000	25-4000	
4-CPP	3307-39-9	25-4000	25-4000	

TABLE 1-continued

Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenoprop	93-72-1	25-4000	25-4000	
3,4-DP	3307-41-3	25-4000	25-4000	

An additional benefit of the AAD-1 gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). See Table 2. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing AAD-1, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, *sorghum*, warm and cool-season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

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.

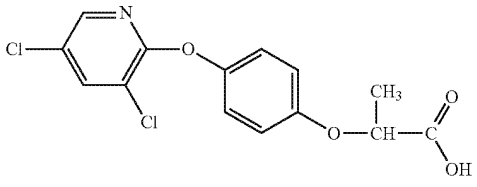
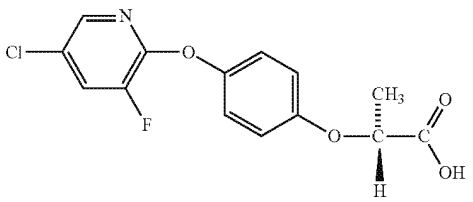
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names.

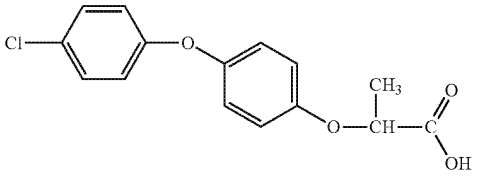
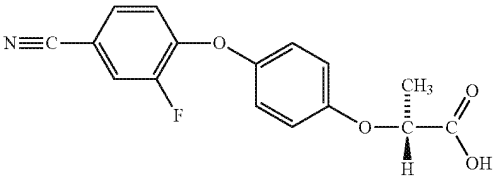
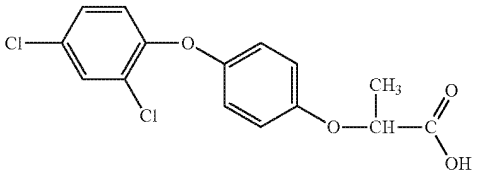
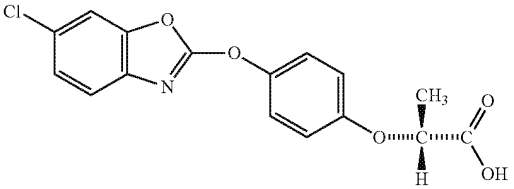
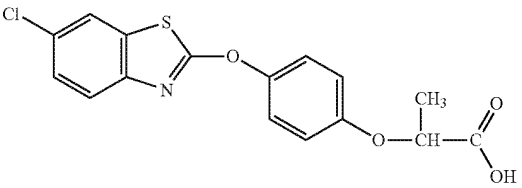
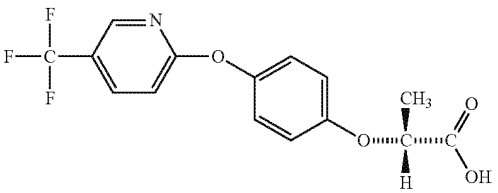
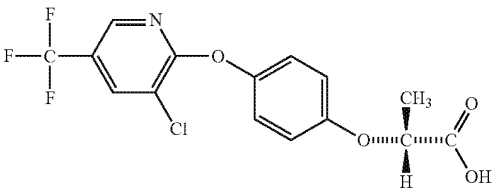
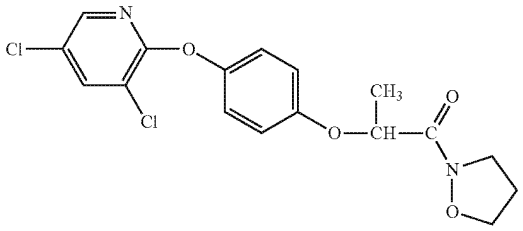
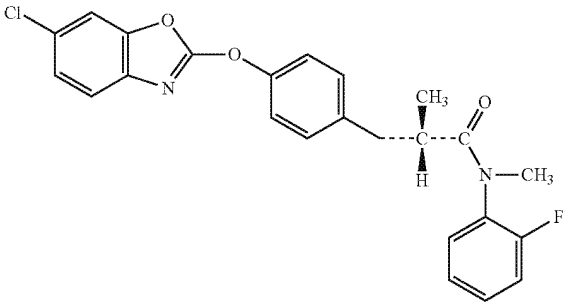
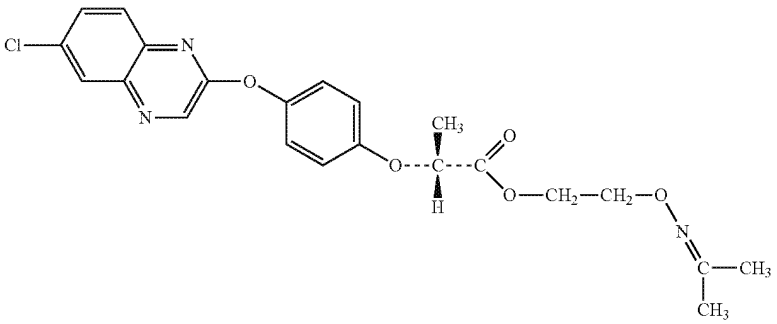
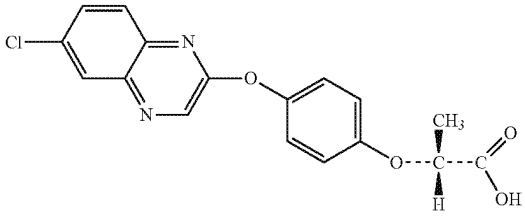
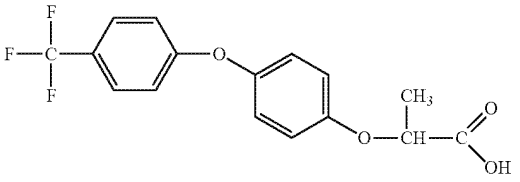
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	

TABLE 2-continued

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
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	

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

pounds 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.

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).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-1 stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

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.

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.

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).

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.

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 (SEQ ID NO: 33) 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.

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. As disclosed herein a peptide of SEQ ID NO: 9 is provided that is capable of degrading 2,4-D, and AOPP herbicides. Mapping the α-ketoglutarate-dependent dioxygenase motif disclosed in the previous paragraph to the sequence of SEQ ID NO: 9 reveals the AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ (SEQ ID NO: 34), wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids.

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.

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.

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.

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.

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.

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. Pat. 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.

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).

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).

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 1 mM 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).

TABLE 3

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

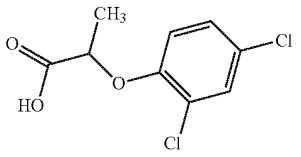
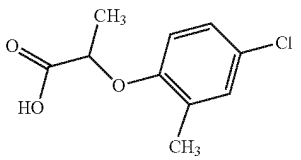
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	117613	(R,S)-dichlorprop	0.566	2.594
	188874	(R,S)-mecoprop	0.341	2.085

TABLE 3-continued

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

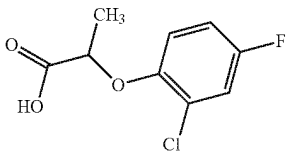
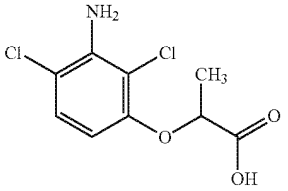
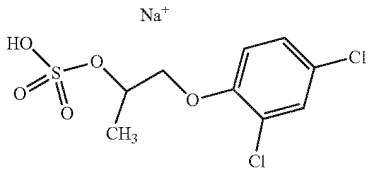
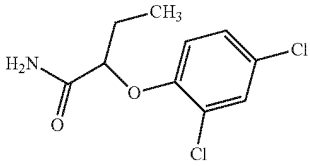
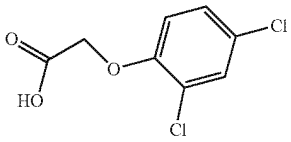
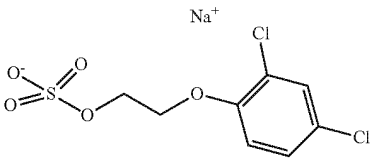
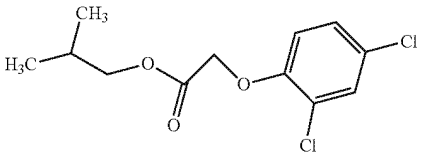
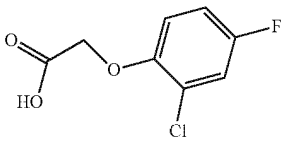
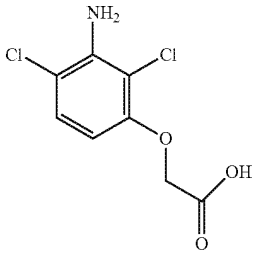
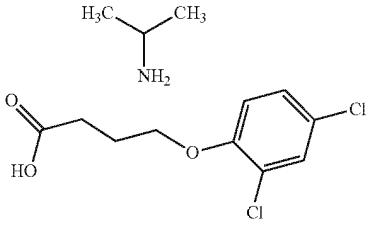
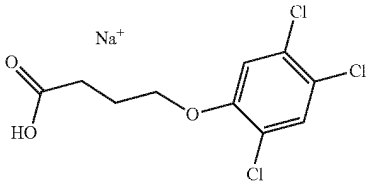
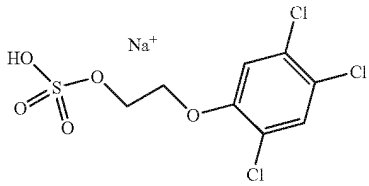
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383
	398166	sesone	0.02	0.177
	190252		0.008	0.211
	124988		0.007	0.058

TABLE 3-continued

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

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	11263526		0.004	0.069
	178577		0.003	0.021
	178587		0.003	0.02
	188527		0.003	0.036

Substrates were assayed at 1 mM in 25 mM MOPS pH 6.8, 200 μM Fe2+, 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.

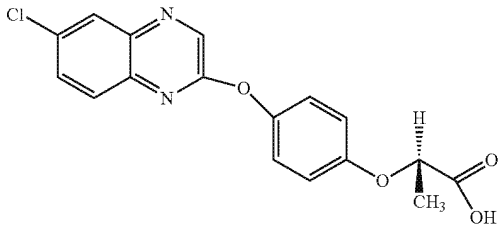
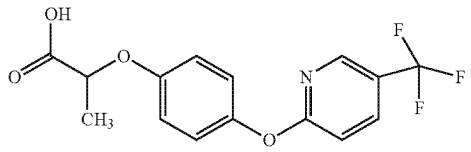
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	18706	(R)-quizalop	0.43	2.1
	67131	(R,S)-fluazifop	0.427	2.17

TABLE 4-continued

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

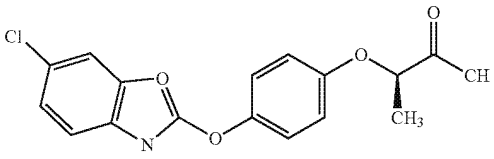
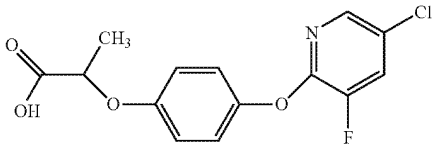
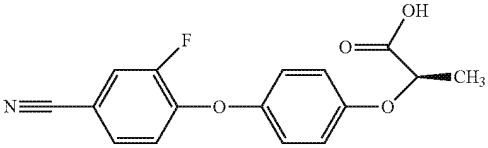
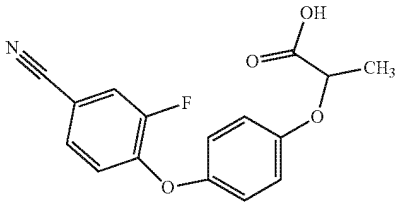
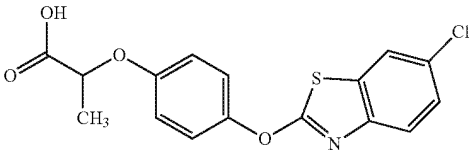
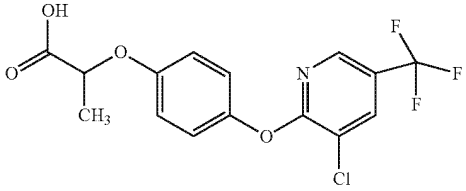
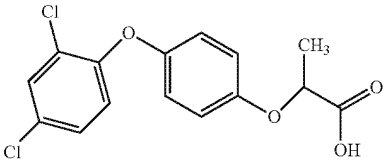
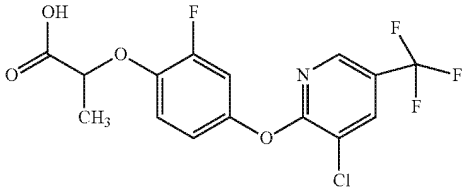
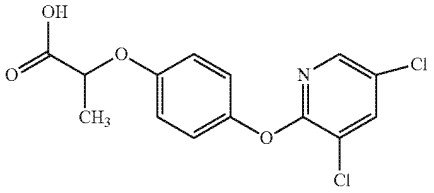
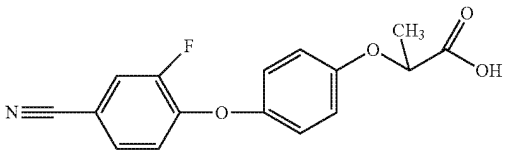
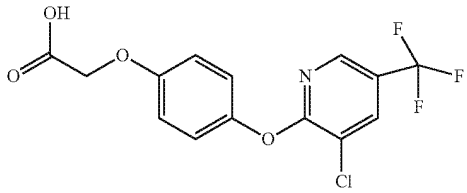
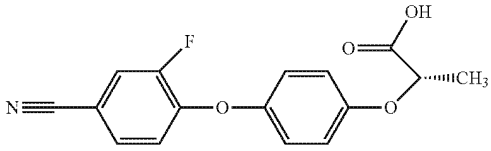
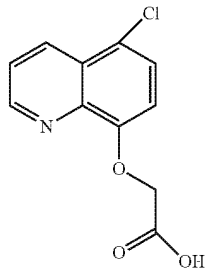
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	11044492 Chiral	(R)-fenoxaprop	0.408	0.597
	34697	(R,S)-clodinofop	0.295	1.98
	14603	(R)-cyhalofop	0.222	1.989
	14623	(R,S)-cyhalofop	0.215	1.815
	62942	(R,S)-fenthiaprop	0.199	1.055
	66905	haloxyfop	0.172	1.63
	460511	(R,S)-diclofop	0.155	1.663
	25646		0.144	1.69

TABLE 4-continued

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

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10x)
	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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.).

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, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2×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 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×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 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×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.

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.

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.

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 6×SSPE, 5×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.

Washes can typically be carried out as follows:

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

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

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C. below the melting temperature (Tm) of the hybrid in 6×SSPE, 5×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).

Washes can typically be out as follows:

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

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

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 2×SSPE, room temperature

Low: 1 or 2×SSPE, 42° C.

Moderate: 0.2× or 1×SSPE, 65° C.

High: 0.1×SSPE, 65° C.

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.

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. Pat. 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.

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.

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.

In addition, U.S. Pat. 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.

"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.

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.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

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.

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.

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

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.

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

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.

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."

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."

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.

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.

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.

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.

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.

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. Pat. No. 5,135,867.

Vectors comprising an AAD-1 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley et al. (1986); and An et al. (1985).

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.

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.

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.

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.

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. No. 4,945,050 to Cornell and U.S. Pat. No. 5,141,131 to Dow-Elanco, 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; U.S. Pat. No. 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 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. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

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.

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

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.

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.

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.

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.

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, 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.

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.

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. No. 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.

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.

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

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.

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.

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.

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' TCTAGAAGGAGATATACC 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' CTCGAGTTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2) (added extra stop codon and Xho I site)].

Twenty microliter reactions were set up as follows: MasterMix 8 μl ea. primer 1 μl (50 pmoles/μl), gDNA 2.5 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 ~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.

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).

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.

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.

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.

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).

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.

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.

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) 250×4.6 mm (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.

2.2—Plate Test Bioassays for Herbicide Degradation.

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.

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.

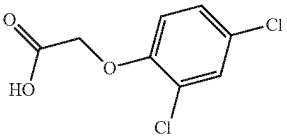
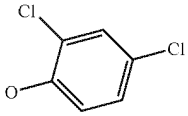
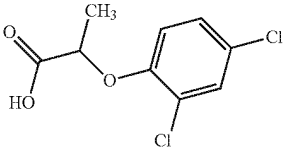
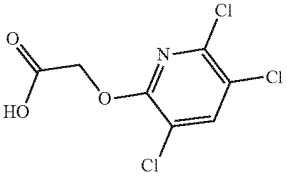
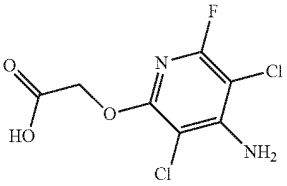
Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4.2H_2O$, 10 μM NaCl, 10 nM $CoCl_2.H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60×15-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.

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.

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

TABLE 6

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

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

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

** GR50 ratio is a measure of the loss of herbicidal activity of enzyme-expressing lysate treatment vs blank vetor 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.

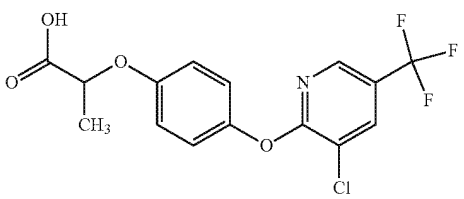
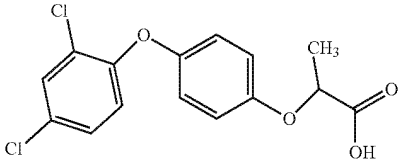
Chemical tested	GR50 (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
Haloxyfop-RS	28	21	520	25	

TABLE 7-continued

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

Chemical tested	GR50 (nM) Chemical alone	Chemical + Blank Vector *	Chemical + AAD-1 v1	GR_{50} ratio **	Structure
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.

2.3—HPLC Results.

From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see FIG. **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.

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 (FIG. **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.

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).

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 (FIG. **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.

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 (FIG. **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.

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 (FIG. **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

3.1—AAD-1 (v1) Assay.

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 FIG. **4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color

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

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.

3.2—Extraction.

Activity of Recombinant AAD-1 (v1) Expressed in *Escherichia coli.*

E. coli cell pellets were resuspended in 0.1 M Tris, pH 7.4+1 mg/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.

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 FIG. **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.

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. FIG. **6**A 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.

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 FIG. **6**B. 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—Additional Substrates of AAD-1 (v1).

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).

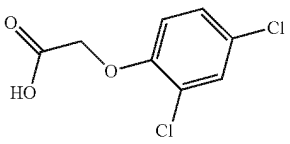
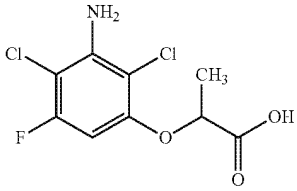
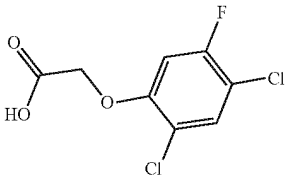
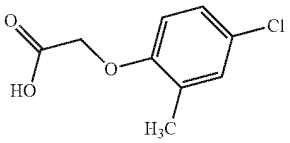
Table 4 shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

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-aminodichlorprop, 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.

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

TABLE 8

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

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

4.2—Kinetic Characterization.

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 FIG. 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).

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

TABLE 9

Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (μM) ± SE	V_{max} (μnol 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

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.

4.3—Additional Substrates for AAD-1 (v1).

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

5.1—Background.

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.

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.

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 MacVector™ program (Accelerys, San Diego, Calif.). 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.aNumber of genes in class given in parentheses.
.sup.bStandard deviations given in parentheses.
.sup.c Combined groups mean ignored in mean calculation

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.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 11. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

TABLE 11

Preferred amino acid codons for proteins expressed in maize

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

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.

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. Pat. No. 5,380,831.

5.2—Rebuild Analysis.

Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native AAD-1 (v1) coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5) was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and Table 12 shows the differences in codon compositions of the native and plant-optimized sequences.

TABLE 12

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

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

5.3—Completion of Binary Vectors.

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).

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).

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.

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 Nod 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.

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).

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.

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*.

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.

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 13. Columns D and H of Table 13 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 13. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

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

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

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 Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H 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

TABLE 13-continued

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

A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
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

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.

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 Tex.).

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, Calif. 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.

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

TABLE 14

Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species* Trans-formed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	----	----	----	Erythro-mycin	----	pat	CsVMV	Agro binary
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	AAD1 v3	CsVMV	Agro binary
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	sacB	HptII	AtUbi3	Agro binary
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	—	HptII	AtUbi3	Agro binary
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	pat	AtUbi10	Aerosol beam
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	----	----	Spectino-mycin	----	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	—	—	Spectino-mycin	—	AHAS v3	OsAct1	Agro Super-binary
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	----	pat	AtUbi11	Agro binary
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	----	----	Erythro-mycin	----	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

6.1—*Arabidopsis thaliana* Growth Conditions.

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).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) 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.

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^2 sec under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2—*Agrobacterium* Transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/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, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

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: 2.4 kV, Pulse length: 5 msec.

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

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, Wis.) per manufacturer's instructions at 0.5× 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.

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. 8700×g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: ½× Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044 µM 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.

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 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), 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.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) 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).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-1f 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.

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, Del.) 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 3200 g ae/ha).

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.

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.

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.

6.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3) (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15). A small lot of AAD-1 (v2) (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

TABLE 15

Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

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

[1]Glufosinate selection scheme: 280 g 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

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 (6× 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.

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

TABLE 16

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

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

TABLE 16-continued

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.

Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev
PAT/Cry1F (transformed control)					
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

Table 17 shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400-1,600 g ae/ha (Table 18). Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some AAD1 (v3) transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first in planta data that AAD-1 (v3) will provide AOPP resistance. No resistance was observed with fluroxypyr (a pyridyloxyacetate auxin) in transformed *Arabidopsis*, consistent with in vitro work using heterologously expressed enzyme.

Table 17. T_1 *Arabidopsis* response to a range of dichloroprop 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.

Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev	Range
AAD-1 v3						
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

TABLE 17-continued

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

Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev	Range
PAT/Cry1F						
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						
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

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.

Averages	% Injury <20%	20-40%	>40%	% Injury Ave	Std Dev	Range
AAD-1 v3						
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						
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

TABLE 18-continued

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.

	% Injury			% Injury	Std	
Averages	<20%	20-40%	>40%	Ave	Dev	Range
Wildtype						
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

6.6—AAD-1 (v3) as a Selectable Marker.

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 (50 g 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.

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.

6.7—Heritability.

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).

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.

6.8—Additional Herbicide Resistance Attributable to AAD-1 in *Arabidopsis*.

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.

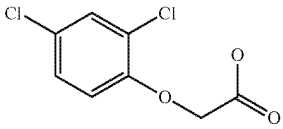
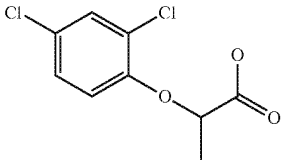
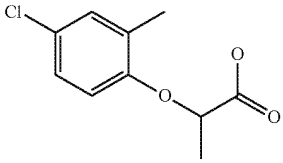
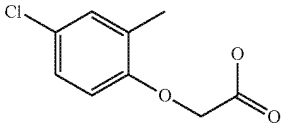
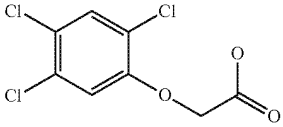
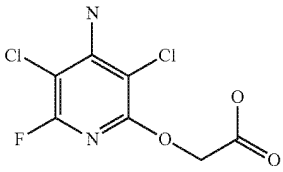
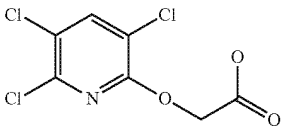
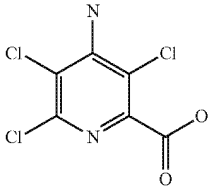
Dose response assays utilized a nutrient media (see below) supplemented with various rates of test compounds. The test compounds were added to the heated media (55° C.) as concentrated solutions in DMSO. Control wells had the appropriate amount of DMSO without any additional compound. The final concentration 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, N.J.). 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).

Assessment of Growth Reduction.

The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in Table 19. A ratio >2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported in planta. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of AAD-1-transformed commercial crops or experimental plant species.

TABLE 19

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

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

6.9—Foliar Applications Herbicide Resistance in AAD-1 *Arabidopsis*.

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.

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.

Herbicide Treatment	Ave % Injury 14DAT: AAD1.01.315.076.T_4 homozygous AAD1 plants	Ave % Injury 14DAT: PatCry1f - Control
Phenoxypropionic auxins		
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		
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
Pyridyloxyacetic auxins		
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

6.10—Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

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. FIGS. **8**A and **8**B 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.

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 (FIG. **9**A). These plants also expressed high level of AAD-1 (v3), from 0.1 to 0.25% of (FIG. **9**B). 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 (FIG. **9**A), 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.

6.11—Molecular Analysis of AAD-1 (v3) *Arabidopsis*.

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 transfor-

mation unit containing PAT and AAD-1 (v3). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

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× 100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of PAT. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

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.

6.12—*Arabidopsis* Transformed with Molecular Stack of AAD-1 (v3) and Glyphosate Resistance Gene.

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 FIG. **10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate+2,4-D treatments applied in tankmix as described in Example 21 and shown for AAD-1 (v3)-transformed corn in Example 8.

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

7.1—Cloning of AAD-1 (v3).

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.

7.2—Callus/Suspension Initiation.

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.

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.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcul-

tured 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.

7.3—Cryopreservation and Thawing of Suspensions.

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.

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 100×25 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.

7.4—Dose Response of Non-Transformed Tissue to Haloxyfop Acid.

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 60×20 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 FIG. **11**.

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 FIG. **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).

7.5—WHISKERS-Mediated Transformation Using Bialaphos Selection.

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

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.

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 60×20 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.

After 1 week, filter papers were transferred to 60×20 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).

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 100×15 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.

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 60×20 mm plates. If sustained growth was evident after approximately

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

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 150×25-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.

7.6—WHISKERS-Mediated Transformation Using Haloxyfop Selection.

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.

After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60×20-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.

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.

7.8—Quantitative Demonstration of In Vitro Tolerance from AAD-1 (v3) Expressing Callus Events from Bialaphos Selection

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	+	+

TABLE 21-continued

Event	PAT Copy #	PCR for AAD-1 (v3) PTU	Callus Western
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	+	+

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 FIG. **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.

7.9—WHISKERS-Mediated Transformation Using Imazethapyr Selection.

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.

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	+++

TABLE 22-continued

AAD-1 Event ID Number	Expression Level (ppm)	Western Blot
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)		++++

7.10—Molecular Analysis: Maize Materials and Methods.

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.

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.

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—CT-CAAGCATATGAATGACCT 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.

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 20×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 1200×100 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

8.1—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 (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 1× lethal dose (~⅛× field dose) up to 8× field

doses (64× 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.

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 8× field dose (64× 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.

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.

FIG. **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.

Table 23 shows data for the responses of selected AAD-1 (v3)-transformed T_0 corn events to three AOPP herbicides applied postemergence.

TABLE 23

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

Construct	Event	Clone	Haloxy-fog* g ae/ha	Cyhalo-fop** g ae/ha	Quizalo-fop*** 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

TABLE 23-continued

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

Construct	Event	Clone	Haloxy-fog* g ae/ha	Cyhalo-fop** g ae/ha	Quizalo-fop*** g ae/ha	% Injury 14 DAT
3404	038	021			70	80
3404	038	022			70	80
			(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 ae/ha)	(lethal dose = 4.4 g ae/ha)	

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

8.2—Field Tolerance of pDAB3404 T_1 corn Plants to Quizalofop 2,4-D, and Glufosinate Herbicides.

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× 5XH571 is of the same parentage as the AAD-1 (v3) event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

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.

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.

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.

8.2.1—Results.

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

TABLE 24

	Treatment (rate) =												
	Quizalofop (140 g ae/ha)		2,4-D amine (1120 g ae/ha)		2,4-D amine (1120 g ae/ha)		Analysis Results Evaluation =			Resistance Segregation Ratios following Liberty Spray		
Event or Hybrid	% Injury 3 WAA (0-100 scale) IN	HI	% Leaning 2 DAA (0-100 scale) IN	HI	Braceroot Deformation 3-4 WAA (0-10 scale) IN	HI	AAD-1 copy number leaf (Southern analysis)	AAD-1 Leaf Western Blot T_0	AAD-1 Leaf ELISA T_0	T_1 Population Average IN	HI	T_2 Population Average 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 ($P > 0.05$)

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.

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.

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).

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 FIG. **16**.

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.

8.3—Stacking of AAD-1 (v3) and PAT with Glyphosate Resistance Genes in Corn.

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.

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.

Plants were allowed to grow until 2-4 leaves had emerged from the whorl. At this point herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. The plants were sprayed with rates of

2,4-D DMA, glyphosate, glufosinate, and various combinations of the three. All applications were formulated in 200 mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

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

		% Injury at 14 DAT	
	Field Rate	Hi II X 5XH751 Ave	RR/PAT/AAD1 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

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.

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 deionized water.

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.

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 (1× field rate, 4× 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 4× lethal dose of quizalofop at the whole plant level.

8.3.3 Expression of AAD-1 (v3) with Respect to Quizalofop Tolerance.

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 FIG. **15**.). The highest tolerance line, event 3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1× 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 1× 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)

9.1—Plant Material.

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.

9.2—Tissue Source.

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.

9.3—Preparation of a Superbinary Vector.

Construction of an *Agrobacterium* construct, pDAB2272, containing the AAD-1 (v3) gene in combination with the AHAS selectable marker gene was accomplished by isolat-

ing 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.

9.4—Bacterial Supply.

All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Pat. No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~1×10^9 cfu/ml) and 100 μM actetosyringone added to the solution.

9.5—Infection and Cocultivation.

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.

9.6—Dose Response Using Immature Embryos.

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 FIG. **17**.

9.7—Selection.

After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit® is used as the chemical selection agent when the AHAS gene is used, based on U.S. Pat. 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.

9.8.—Regeneration and Seed Production.

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.

9.9— Event Recovery and Analysis Particulars; Whole Plant Screening of T_0 Corn Lineages Containing AAD-1 (v3) and AHAS (pDAS1421).

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

	In vitro Selection agent	Southern Copy # (callus)	Western Blot (AAD-1) T_0	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop	
Corn event	(nM)	AAD-1	Callus	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

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

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 (24,000 g) 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

Plasmid pDAB3203 containing the AAD-1 (v1) gene was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C. with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C. with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000×g for 20 mM to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

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.

Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) 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).

11.1—Extracting AAD-1 (v3) from Plant Leaves.

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 5,000×g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

11.2—Bradford Assay.

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).

11.3—Enzyme Linked Immuno-Sorbent Assay (ELISA).

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).

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

11.4—Western Blotting Analysis.

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

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, Fla.) 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.

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 $\mu E/m^2s^1$).

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.

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

TABLE 27

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

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

TABLE 27-continued

Tobacco T0 events transformed with pDAB721 (AAD-1(v3) + PAT).				
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 (14 DAT)
	Low	>50% injury
	Medium	20-50% injury
	High	<20% injury
	Variable	inconsistent

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.

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 (Minis 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.

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.

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 1.5× 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 (~6× 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).

Verification of High 2,4-D Tolerance.

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 187 L/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 200 mM 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 1× field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by AAD-1 (v3), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

TABLE 28

Segregating AAD-1 T_1 tobacco plants' response to phenoxy auxin herbicides.			
Herbicide	KY160 - Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
	Average % Injury of Replicates 14 DAT		
560 g ae/ha 2,4-D DMA	75	5	0
1120 g ae/ha 2,4-D DMA	80	5	2
2240 g ae/ha 2,4-D DMA	90	5	0
4480 g ae/ha 2,4-D DMA	95	5	5
560 g ae/ha R-dichlorprop	70	5	0
1120 g ae/ha R-dichlorprop	75	5	0
2240 g ae/ha R-dichlorprop	88	5	0
4480 g ae/ha R-dichlorprop	95	10	5
560 g ae/ha 2,4-D DMA/R-dichlorprop	80	5	5
1120 g ae/ha 2,4-D DMA/R-dichlorprop	80	10	10
2240 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15
4480 g ae/ha 2,4-D DMA/R-dichlorprop	95	15	15

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.

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

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.

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).

13.1—Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

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).

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.

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.

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 100×25 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).

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(2-1N-morpholinolethane 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.

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.

Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from 10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

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.

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.

13.2—Transformation Method 2: "No-Shake" *Agrobacterium* Mediated Transformation of Non-Regenerable Soybean Suspension Cells.

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.

Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100×25 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.

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 100×15 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 5× concentration of glufosinate ammonium and were maintained for up to 4 months.

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 (FIG. **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%

13.3—Transformation Method 3: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

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

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 ½ B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

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 ½ B5G which contained all ingredients of B5G medium at half strength.

TABLE 30

Growth Media for Soybean

	Ingredients in 1 Liter		
	B1-30	B3	B5G
Ms Salts	4.43 g	4.43 g	
B5 Salts			3.19 g
NaEDTA	37.3 mg	37.3 mg	37.3 mg
2,4-D	30 mg		

TABLE 30-continued

Growth Media for Soybean

	Ingredients in 1 Liter		
	B1-30	B3	B5G
Activated charcoal		5 g	
Phytagar	8 g	8 g	
Gelrite			2 g
pH	5.8	5.8	5.8

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

14.1—Cotton Transformation Protocol.

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.

Cotyledon segments (~5 mm) 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.

Agrobacterium Preparation.

Inoculate 35 ml 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.

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.

Larger, well-developed embryos are isolated and transferred to DK media (Table 31) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

TABLE 31

Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1 mM)		1 ml	1 ml	1 ml	4.6 ml		0.5 ml	
2,4-D (1 mM)		1 ml	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 (10 mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

14.2—Experiment Specifics.

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.

14.3—Callus Analysis Results.

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
1	2	pos	***
2	1	pos	***
3	1	pos	***
4	2	pos	***
5	1	pos	***
6	1	pos	neg
7	1	pos	****
8	1	pos	*****
9	2	pos	*
10	1	pos	*
11	1	pos	*****
12	1	pos	*
13	1	pos	*

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
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	**
43	1	pos	*****
44	low DNA	pos	*****
45	1	pos	*****
46	2	pos	*****
47	1	pos	***
48	1	pos	***
49	3	faint pos	neg
50	1	pos	****
51	4	pos	neg
52	2	pos	****
53	1	pos	***
54	1	pos	****
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	***

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
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	*
standard	AAD1	5 ug/ml	*****
standard	AAD1	0.5 ug/ml	**

14.4—Plant Regeneration.

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.

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

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.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform AAD-1 (v3), for example, into these and other plants, including but not limited to Maize (Gramineae *Zea mays*), Wheat (Pooideae *Triticum* spp.), Rice (Gramineae *Oryza* spp. and *Zizania* spp.), Barley (Pooideae *Hordeum* spp.), Cotton (*Abroma* Dicotyledoneae *Abroma augusta*, and *malvaceae gossypium* spp.), Soybean (*Soya leguminosae Glycine max*), Sugar beet (Chenopodiaceae *Beta vulgaris altissima*), Sugar cane (*Arenga pinnata*), Tomato (Solanaceae *Lycopersicon esculentum* and other spp., *Physalis ixocarpa, Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (Pooideae *Secale* spp.), Peppers (Solanaceae *Capsicum annuum, sinense*, and *frutescens*), Lettuce (Compositae *Lactuca sativa, perennis*, and *pulchella*), Cabbage, Celery (Umbelliferae *Apium graveolens*), Eggplant (Solanaceae *Solanum melongena*), *Sorghum* (all *Sorghum* species), Alfalfa (Leguminosae *Medicago sativum*), Carrot (Umbelliferae *Daucus carota sativa*), Beans (Leguminosae *Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (Leguminosae *Pisum, Vigna*, and *Tetragonolobus* spp.), Sunflower (Compositae *Helianthus annuus*), Squash (Dicotyledoneae *Cucurbita* spp.), Cucumber (Dicotyledoneae genera), Tobacco (Solanaceae *Nicotiana* spp.), *Arabidopsis* (Cruciferae *Arabidopsis thaliana*), Turfgrass (*Lolium, Agrostis*, and other families), and Clover (Leguminosae). Such plants, with AAD-1 (v3) genes, for example, are included in the subject invention.

Example 16—Stacking AAD-1 (v3) with AHAS Herbicide Resistance Gene

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

17.1—AAD-2 (v1) 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 (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.

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

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

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.

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.

17.2—Completion of AAD-2 (v1) Binary Vector.

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.*

17.3—Comparison of Substrate Specificities of AAD-2 (v1) and AAD-1 (v1)

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. FIG. **22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D=dichlorprop>(R,S)-haloxyfop>>(R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. Table 33 shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

TABLE 33

Comparison of AAD1 and AAD2 activity on various substrates.

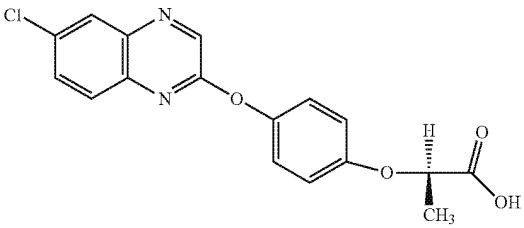
STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
	18706	quizalofop	R	0.27	0.01

TABLE 33-continued

Comparison of AAD1 and AAD2 activity on various substrates.

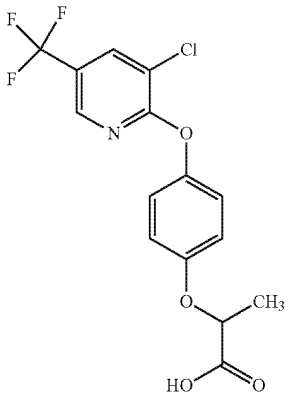
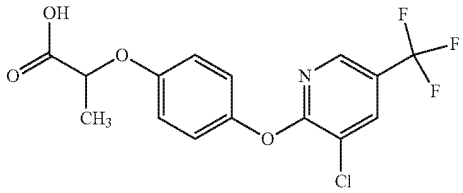
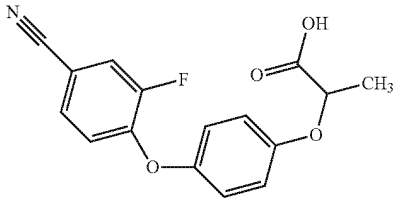
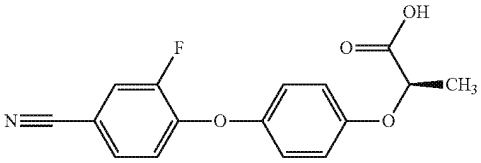
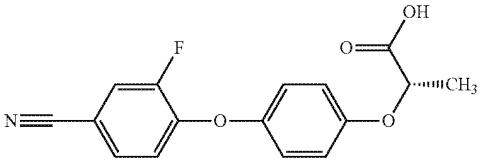
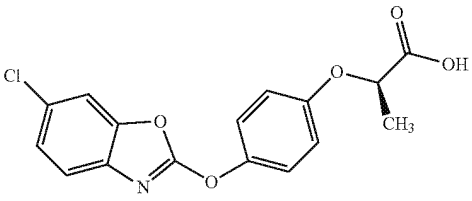
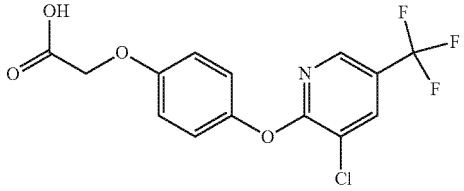
STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15
	Chiral 11044492	fenoxaprop	R	0.14	0

TABLE 33-continued

Comparison of AAD1 and AAD2 activity on various substrates.

STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
	43865	haloxyfop-	—	0	22

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).

The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. See FIG. **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.

17.4—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 (v1) gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T_1 seed (40,000 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.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) 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).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-1f stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C., 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural+supplemental light) at least 1 day prior to testing for the ability of AAD-1 (v3), AAD-1 (v2), or AAD-2 (v1) to provide phenoxy auxin herbicide resistance.

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, Del.) 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).

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 trans-

formed 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).

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.

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 FIG. **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).

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 FIG. **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

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With AAD-1 (v3) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosinate+2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed.

Example 19—in-Crop Use of Phenoxy Auxins Herbicides in AAD-1 (v3) Only Transformed Soybeans, Cotton, and Other Dicot Crops

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).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

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.

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

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-1 (v3) would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. AAD-1 (v3)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, et al. could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of AAD-1 (v3)-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3) transformed monocot crop (e.g., corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

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

The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, *Petunia*, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-1 (v3) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a GT trait are stacked in any monocot or dicot crop species:

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

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AAD-1 (v3)+GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. An AOPP herbicide like quizalofop at 10-200 g ae/ha (preferably 20-100 g ae/ha and more preferably 20-35 g ae/ha), could be for more robust grass weed control and/or for delaying the development of glyphosate resistant grasses. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D.

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

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).

By stacking AAD-1 (v3) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

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

i) Inherently tolerant grass weed species (e.g., *Echinochloa* spp or *Sorghum* spp) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

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

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like AHAS et al. Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking AAD-1 (v3) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

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

i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.

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

iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

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

24.1—Media Description.

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 100×20 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).

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 hygro-

mycin 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 (½MSH50).

24.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa L. japonica* cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week prior to storage at 4° C.

24.3—Microprojectile Bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (BioRad, 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).

24.4—Tolerance Testing.

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

24.5—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 scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/μl.

24.6—Southern Blot Analysis.

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×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 1200×100 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. 20 mM 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 Biosys-

tems), 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.

The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in Table 36.

TABLE 36

Southern Results.

Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049 bp
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

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.

24.7—Western Data

Sample preparation and analysis conditions were as described previously. Five transgenic rice lines and 1 non-transgenic control were analyzed for AAD-1 expression using ELISA and Western blot. AAD-1 was detected in four lines (63-1A, 63-1F, 63-4B and 63-4D) but not in line 63-1C or the control plant. Expression levels ranged from 15.6 to 183 ppm of total soluble protein. A summary of the results is presented in Table 37.

TABLE 37

Lane	Sample Name	TSP (µg/mL)	ELISA [AAD1] (ng/mL)	ELISA Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	–
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	–
7	AAD1 Standard (0.5 µg/mL)				+++
8	AAD1 Standard (5.0 µg/mL)				+++++

Example 25—Turf Grass Transformation Procedures

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).

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.

25.1—Seed Preparation for Embryogenic Callus Induction.

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).

25.2—Embryogenic Callus Induction.

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.

25.3—*Agrobacterium* Infection and Co-Cultivation.

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.

25.4—Resting Stage and *Agrobacterium* Control.

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.

25.5—Selection and Potential Transgenic Colony Identification.

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.

25.6—Regeneration of Transgenic Plants.

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).

25.7—Root Induction and Transfer to Greenhouse.

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.

25.8—Vernalization and Out-Crossing of Transgenic Plants.

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.

25.9—Other Target Grasses.

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

26.1—Canola Transformation.

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.

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.

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.

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

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).

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.

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.

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.

26.2—"Molecular Analysis": Canola Materials and Methods

26.2.1—Tissue Harvesting DNA Isolation and Quantification.

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

26.2.2—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 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.

26.3—ELISA.

Using established ELISA described in previous section, AAD-1 protein was detected in 10 different canola transformation events. Expression levels ranged from 150 to over 1000 ppm of total soluble protein (TSP). Three different untransformed calli samples were tested in parallel with little signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. A summary of the results is presented in Table 38.

TABLE 38

Expression of AAD1 in Canola calli.

Sample #	Weight (mg)	[TSP] (µg/mL)	[AAD1] (ng/mL)	Expression (ppm TSP)	PCR for AAD1
1	114	757.02	119.36	157.67	+
2	55	839.79	131.84	156.99	+
3	53	724.41	202.12	279.01	+
4	52	629.01	284.89	452.92	+
5	55	521.75	175.88	337.08	+
6	61	707.69	74.24	153.71	+
7	51	642.02	559.11	1026.73	+
8	65	707.69	270.73	382.56	+
9	51	642.02	197.90	308.25	+
10	51	1417.42	220.63	156.66	+
Control 1	53	2424.67	18.67	7.70	−
Control 2	61	2549.60	35.00	13.73	−
Control 3	59	2374.41	22.79	9.60	−

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Zhao, Z. Y., T. Cai, L. Tagliani, M. Miller, N. Wang, H. Pang, M. Rudert, S. Schroeder, D. Hondred, J. Seltzer, and D. Pierce. 2000. *Agrobacterium*-mediated *Sorghum* transformation, Plant Mol. Biol. 44:789-798.

Zipper, C., M. Bunk, A. Zehnder, H. Kohler. 1998. Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(RS)-2-(2,4-dichlorophenoxy)propanoic acid] by *Sphingobium herbicidovorans* MH. J. Bact. 13:3368-3374

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 35

<210> SEQ ID NO 1
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer used to amplify the rdpA/AAD-1 (v1) gene

<400> SEQUENCE: 1

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<210> SEQ ID NO 2
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer used to amplify the rdpA/AAD-1

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(v1) gene

<400> SEQUENCE: 2

ctcgagttac tagcgcgccg ggcgcacgcc accgaccg 38

<210> SEQ ID NO 3
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<212> TYPE: DNA
<213> ORGANISM: Sphingobium herbicidovorans
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(18)
<223> OTHER INFORMATION: Primer linker
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (907)..(915)
<223> OTHER INFORMATION: Primer linker

<400> SEQUENCE: 3

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atcgcggtcc agccgctgac cggcgtcctg ggcgccgaga tcaccggcgt cgacctgcgc 120

gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttccacac ttaccaggtc 180

atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc 240

ggccccgtcg atcccgtgcc cctgctcaag agcatcgaag ggtatccaga ggtgcagatg 300

atccgccgcg aagccaacga aagcgggcgt gtgatcggtg aygactggca caccgacagc 360

accttcctgg acgcaccgcc ggccgccgtg gtgatgcgcg cgatcgacgt gcccgagcat 420

ggcggcgaca ccggttttct gagcatgtac accgcgtggg agacgctgtc gcccaccatg 480

caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggctcgctc 540

taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg 600

ggcgaccgtg aaaccgtgca ccccctggtg gtgacccatc cgggcagcgg ccgcaagggc 660

ctgtacgtga accaggtcta ttgccagcgc atcgagggca tgaccgatgc cgaaagcaaa 720

ccgctgctgc agttcctgta cgagcatgcg acacggttcg atttcacctg ccgcgtgcgc 780

tggaagaagg accaggtcct ggtctgggac aacctgtgca cgatgcaccg ggccgtaccc 840

gactacgcgg gcaagttccg ctacctgacg cgcaccacgg tcggtggcgt gcgcccggcg 900

cgctagtaac tcgag 915

<210> SEQ ID NO 4
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 (v2) primary sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: primer linker
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (891)..(897)
<223> OTHER INFORMATION: primer linker

<400> SEQUENCE: 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

-continued

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> SEQ ID NO 5
<211> LENGTH: 919
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 (v3) primary sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: primer linker
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (6)..(8)
<223> OTHER INFORMATION: additional alanine codon (GCT)
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (894)..(919)
<223> OTHER INFORMATION: primer linker

<400> SEQUENCE: 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> SEQ ID NO 6

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<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: rdpA(ncoI) 5' primer

<400> SEQUENCE: 6

cccatggctg ctgcactgtc ccccctctcc 30

<210> SEQ ID NO 7
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 3'saci primer

<400> SEQUENCE: 7

gagctcacta gcgcgccggg cgcacgccac cga 33

<210> SEQ ID NO 8
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: BstEII/ Del NotI 5' primer

<400> SEQUENCE: 8

tggtggtgac ccatccgggc agcggctgca agggcc 36

<210> SEQ ID NO 9
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Sphingobium herbicidovorans

<400> SEQUENCE: 9

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

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

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

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

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

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

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

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

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

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

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

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

Asp Ala Gly Asp Arg Glu Thr Val His Pro Leu Val Val Thr His Pro

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        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> SEQ ID NO 10
<211> LENGTH: 295
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v2 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Different from v1
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (212)..(212)
<223> OTHER INFORMATION: Different from v1

<400> SEQUENCE: 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

Gly Ser Gly Cys Lys Gly Leu Tyr Val Asn Gln Val Tyr Cys Gln Arg
    210                 215                 220
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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> SEQ ID NO 11
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 v3 Translation
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (2)..(3)
<223> OTHER INFORMATION: Different from V1

<400> SEQUENCE: 11

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Lys Lys Asp Gln Val Leu Val Trp Asp Asn Leu Cys Thr Met His Arg
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            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> SEQ ID NO 12
<211> LENGTH: 888
<212> TYPE: DNA
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 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> SEQ ID NO 13
<211> LENGTH: 289
<212> TYPE: PRT
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

<400> SEQUENCE: 13

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

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

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

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

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

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

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

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

Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe
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    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> SEQ ID NO 14
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: brjap 5'(speI)

<400> SEQUENCE: 14

actagtaaca aagaaggaga tataccatga cgat 34

<210> SEQ ID NO 15
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: br jap 3' (xhoI)

<400> SEQUENCE: 15

ttctcgagct atcactccgc cgcctgctgc tgc 33

<210> SEQ ID NO 16
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 forward primer

<400> SEQUENCE: 16

gtaaaacgac ggccagt 17

<210> SEQ ID NO 17
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: M13 reverse primer

<400> SEQUENCE: 17

caggaaacag ctatgac 17

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<210> SEQ ID NO 18
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: NcoI of Brady

<400> SEQUENCE: 18

tataccacat gtcgatcgcc atccggcagc tt 32

<210> SEQ ID NO 19
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SacI of Brady

<400> SEQUENCE: 19

gagctcctat cactccgccg cctgctgctg cac 33

<210> SEQ ID NO 20
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Glycine max

<400> SEQUENCE: 20

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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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> SEQ ID NO 21
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Doubly mutated Soybean EPSPS protein: threonine
      183 converted to isoleucine; proline 187 converted to serine

<400> SEQUENCE: 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
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                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
        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
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Pro Asp Tyr Phe Glu Val Leu Glu Arg Leu Thr Lys His
515 520 525

<210> SEQ ID NO 22
<211> LENGTH: 1604
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Soybean-biased DNA sequence encoding doubly-mutated EPSPS disclosed in SEQ ID NO:21, with added sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1575)
<223> OTHER INFORMATION: Coding sequence of soybean-biased DNA sequence encoding the EPSPS protien of SEQ ID NO:21
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1576)..(1578)
<223> OTHER INFORMATION: Translation terminator tga
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1579)..(1604)
<223> OTHER INFORMATION: Sequence included to introduce translation stop codons in all six open reading frames, and to introduce a SacI restriction enzyme recognition site for cloning purposes

<400> SEQUENCE: 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

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actatcaaag accctgggtg cacaaggaag acatttccag actactttga agttttggag 1560

aggttgacaa agcactgagt agttagctta atcacctaga gctc 1604

<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 5-3

<400> SEQUENCE: 23

agataccctt ggttggttgc 20

<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 3-3

<400> SEQUENCE: 24

cagatggatc gtttggaagg 20

<210> SEQ ID NO 25
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU forward primer

<400> SEQUENCE: 25

ataatgccag cctgttaaac gcc 23

<210> SEQ ID NO 26
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU reverse primer

<400> SEQUENCE: 26

ctcaagcata tgaatgacct cga 23

<210> SEQ ID NO 27
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for Coding Region PCR AAD-1 (RdpAcodF)

<400> SEQUENCE: 27

atggctcatg ctgccctcag cc 22

<210> SEQ ID NO 28
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for Coding Region PCR AAD-1 (RdpAcodR)

<400> SEQUENCE: 28

cgggcaggcc taactccacc aa 22

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<210> SEQ ID NO 29
<211> LENGTH: 932
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-2 v2 plant-optimized nucleotide

<400> SEQUENCE: 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

<210> SEQ ID NO 30
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Translated AAD-2 v2 protein

<400> SEQUENCE: 30

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

Val Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg
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

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

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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
            260                 265                 270

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

Thr Val Gln Gln Gln Ala Ala Glu
    290                 295


<210> SEQ ID NO 31
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 forward primer

<400> SEQUENCE: 31

atggctcatg ctgccctcag cc                                              22


<210> SEQ ID NO 32
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 reverse primer

<400> SEQUENCE: 32

gggcaggcct aactccacca a                                               21


<210> SEQ ID NO 33
<211> LENGTH: 84
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: alpha-ketoglutarate-dependent dioxygenase motif
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: Xaa at position 3 is aspartic acid or glutamic
      acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(7)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa at position 8 is threonine or serine
<220> FEATURE:
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<221> NAME/KEY: misc_feature
<222> LOCATION: (9)..(78)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
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<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 33

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His Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1               5                   10                  15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
            20                  25                  30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        35                  40                  45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
    50                  55                  60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa His Xaa
65                  70                  75                  80

Xaa Xaa Xaa Arg
```

<210> SEQ ID NO 34
<211> LENGTH: 171
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 motif
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(27)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(159)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (161)..(170)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 34

```
His Xaa Asp Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1               5                   10                  15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Thr Xaa Xaa Xaa Xaa
            20                  25                  30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        35                  40                  45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
    50                  55                  60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
65                  70                  75                  80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                85                  90                  95

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
            100                 105                 110

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        115                 120                 125

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
    130                 135                 140
```

-continued

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa His
145 150 155 160

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Arg
165 170

<210> SEQ ID NO 35
<211> LENGTH: 175
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 motif extended
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(27)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(159)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (161)..(170)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (172)..(174)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 35

His Xaa Asp Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Thr Xaa Xaa Xaa Xaa
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
50 55 60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
65 70 75 80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
85 90 95

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
100 105 110

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
115 120 125

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
130 135 140

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa His
145 150 155 160

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Arg Xaa Xaa Xaa Arg
165 170 175

The invention claimed is:

1. A transgenic maize plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide which are aryloxyalkanoate herbicides, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of: $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ (SEQ ID NO: 34), wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids, wherein said AAD-1 protein when expressed in a maize plant cell renders said maize plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as compared to an untransformed maize plant cell.

2. The plant cell of claim **1** wherein said AAD-1 motif has the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R(X)_{282-284}R$ (SEQ ID NO: 35), wherein $(X)_{282-284}$ represents a sequence of 3 amino acids.

3. A transgenic maize plant comprising a plurality of the maize plant cell of claim **1**, wherein expression of said polynucleotide renders said plant tolerant to said aryloxyalkanoate herbicide.

4. The plant of claim **3** wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

5. The plant of claim **3** wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

6. The plant of claim **3** wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

7. The plant of claim **3** wherein said plant further comprises a second herbicide resistance gene.

8. The plant of claim **7** wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

9. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim **3**, 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 (R)-aryloxyphenoxypropionate herbicide.

10. The method of claim **9** wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

11. The method of claim **9** wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

12. The method of claim **9** wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

13. The method of claim **9** wherein said method comprises applying a second herbicide.

14. The method of claim **13** wherein said first herbicide and said second herbicide are applied sequentially.

15. The method of claim **13** wherein said first herbicide and said second herbicide are applied concurrently.

16. The method of claim **13** wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

17. The method of claim **13** wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

18. The method of claim **13**, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

19. The method of claim **13**, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

20. The method of claim **13** wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

21. The method of claim **20** wherein said second gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

22. The method of claim **13** wherein said method further comprises applying a third herbicide.

23. The method of claim **22**, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

24. The method of claim **23** wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

25. A seed comprising the plant cell of claim **1**.

26. A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim **25** in said field within 14 days of applying said aryloxyalkanoate herbicide.

27. A plant grown from the seed of claim **25** comprising said polynucleotide.

28. A part, progeny, or asexual propagate of the plant of claim **27**, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

29. An (R)-aryloxyphenoxypropionate herbicide tolerant transgenic maize plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of: $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ (SEQ ID NO: 34), wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids, wherein said motif has 90% sequence identity with corresponding amino acids of position 111 to 281 of SEQ ID NO: 9, and

wherein said AAD-1 protein when expressed in a maize plant cell renders said maize plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxy-propionate herbicide, as compared to an untransformed maize plant cell.

* * * * *

EXHIBIT H

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June 2, 2022

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(54) **HERBICIDE RESISTANCE GENES**

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(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.

29 Claims, 24 Drawing Sheets

Specification includes a Sequence Listing.

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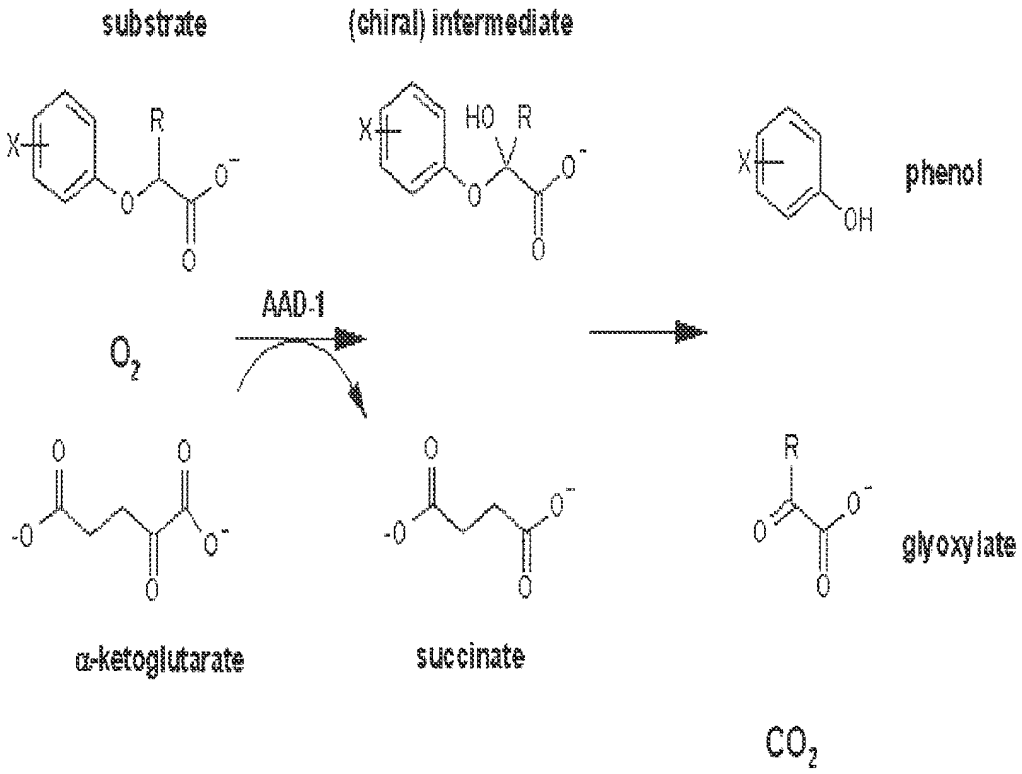
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- Addition of O_2 is *stereospecific*
- Breakdown of intermediate to phenol + glyoxylate is spontaneous

Fig. 1

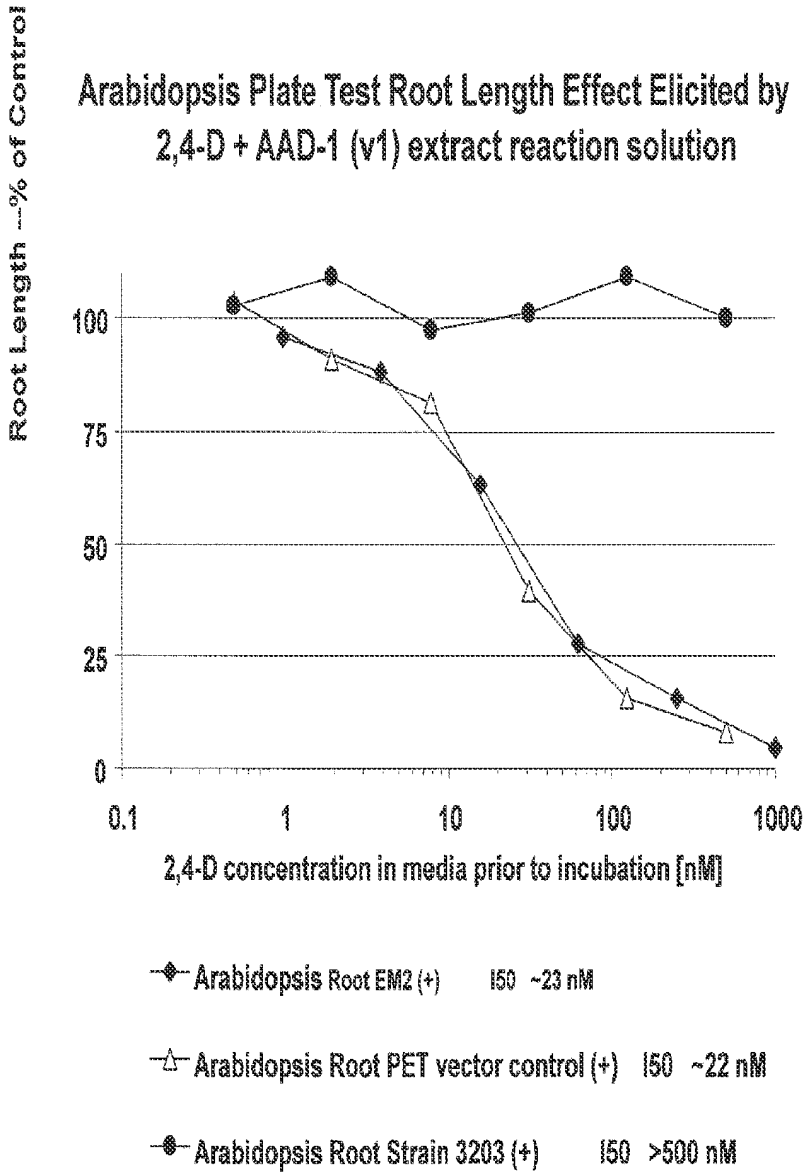

Arabidopsis Plate Test Root Length Effect Elicited by
2,4-D + AAD-1 (v1) extract reaction solution
Root Length --% of Control
100
75
50
25
0
0.1
1
10
100
1000
2,4-D concentration in media prior to incubation [nM]
Arabidopsis Root EM2 (+) I50 ~23 nM
Arabidopsis Root PET vector control (+) I50 ~22 nM
Arabidopsis Root Strain 3203 (+) I50 >500 nM

Fig. 2

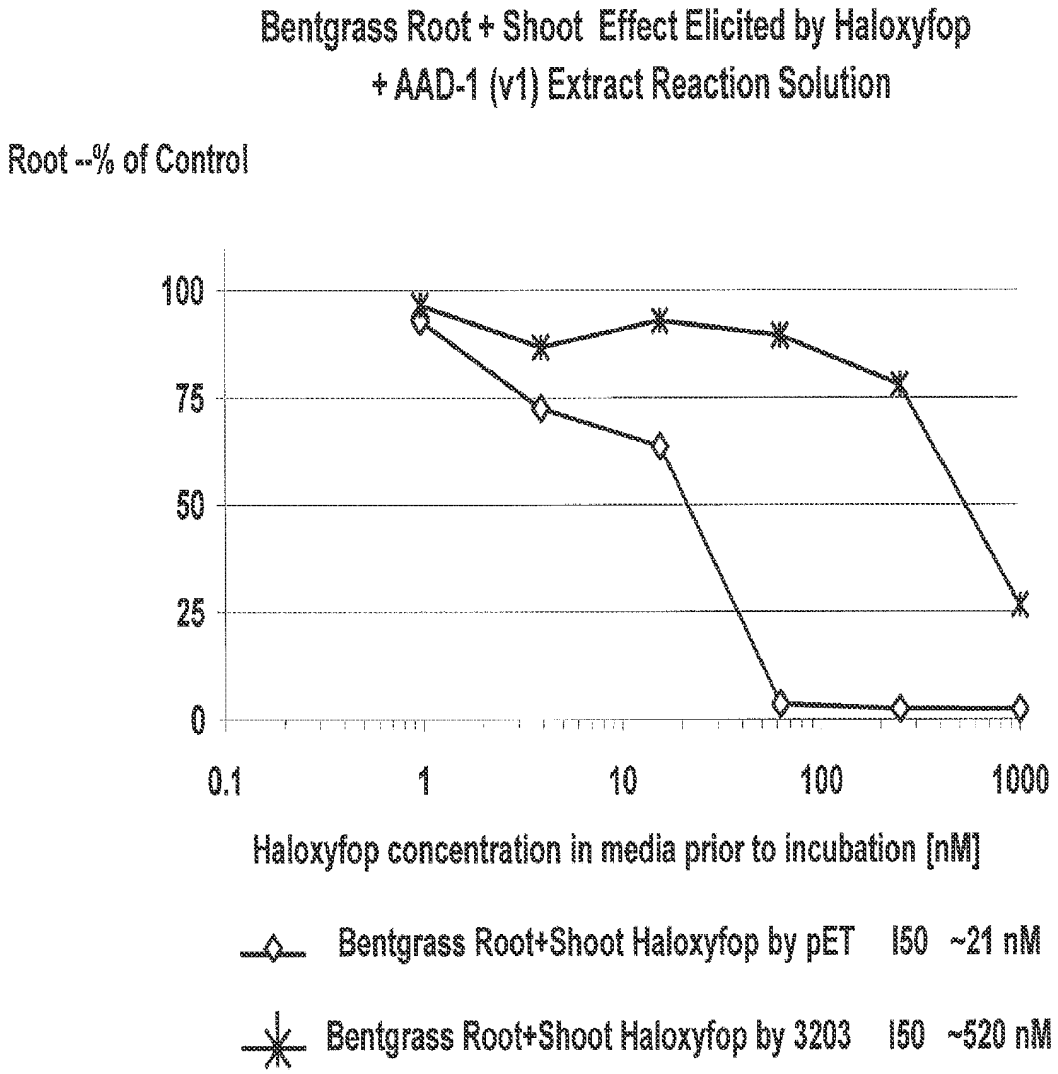

Bentgrass Root + Shoot Effect Elicited by Haloxyfop
+ AAD-1 (v1) Extract Reaction Solution
Root --% of Control
100
75
50
25
0
0.1
1
10
100
1000
Haloxyfop concentration in media prior to incubation [nM]
Bentgrass Root+Shoot Haloxyfop by pET I50 ~21 nM
Bentgrass Root+Shoot Haloxyfop by 3203 I50 ~520 nM

Fig. 3

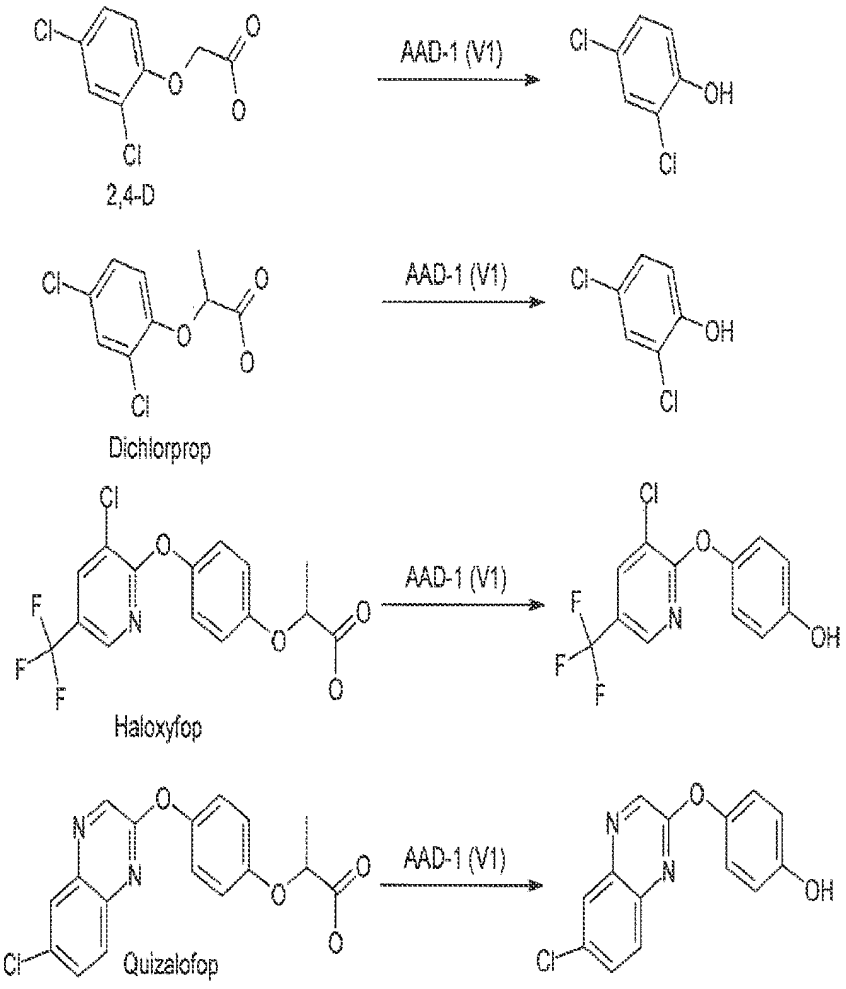

AAD-1 (V1)
2,4-D
AAD-1 (V1)
Dichlorprop
AAD-1 (V1)
Haloxyfop
AAD-1 (V1)
Quizalofop

Fig. 4

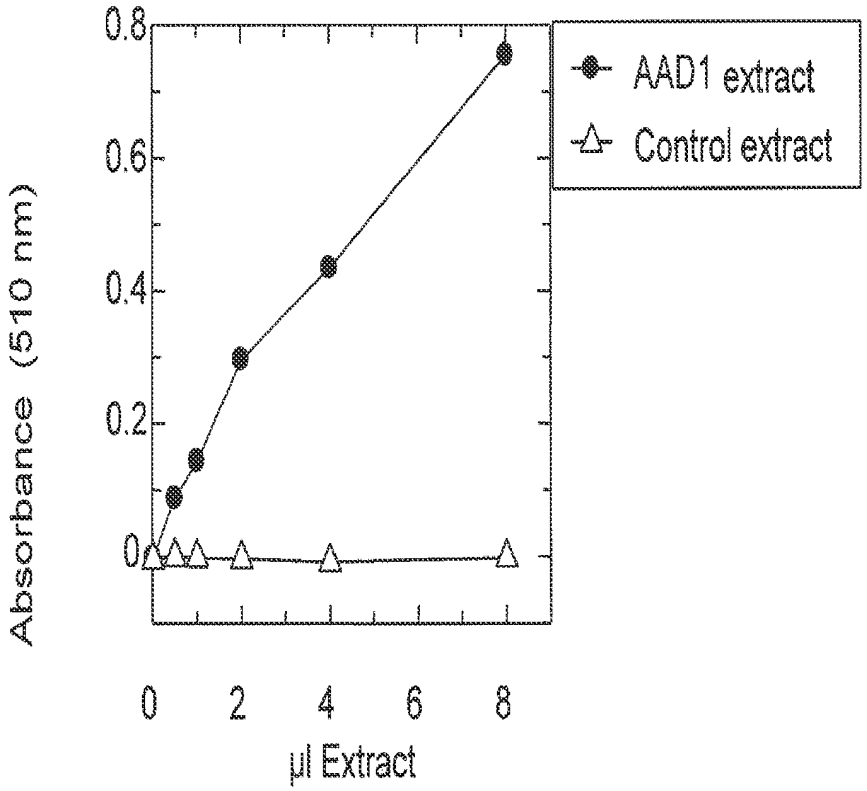

0.8
0.6
0.4
0.2
0
Absorbance (510 nm)
0
2
4
6
8
µl Extract
AAD1 extract
Control extract

Fig. 5

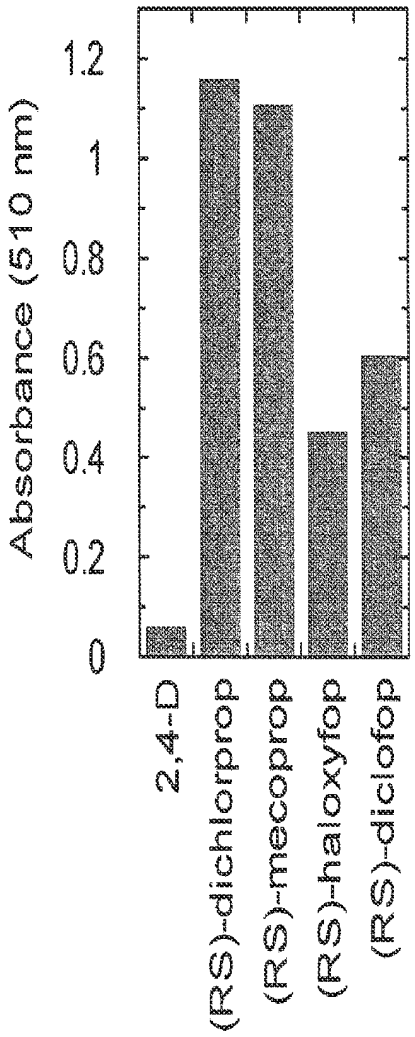

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
2,4-D
(RS)-dichlorprop
(RS)-mecoprop
(RS)-haloxyfop
(RS)-diclofop

Fig. 6A

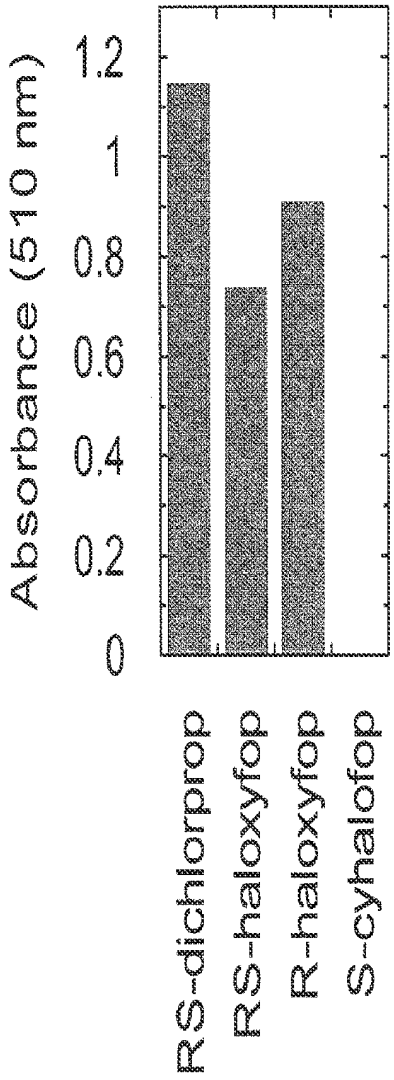

Absorbance (510 nm)
0
0.2
0.4
0.6
0.8
1
1.2
RS-dichlorprop
RS-haloxyfop
R-haloxyfop
S-cyhalofop

Fig. 6B

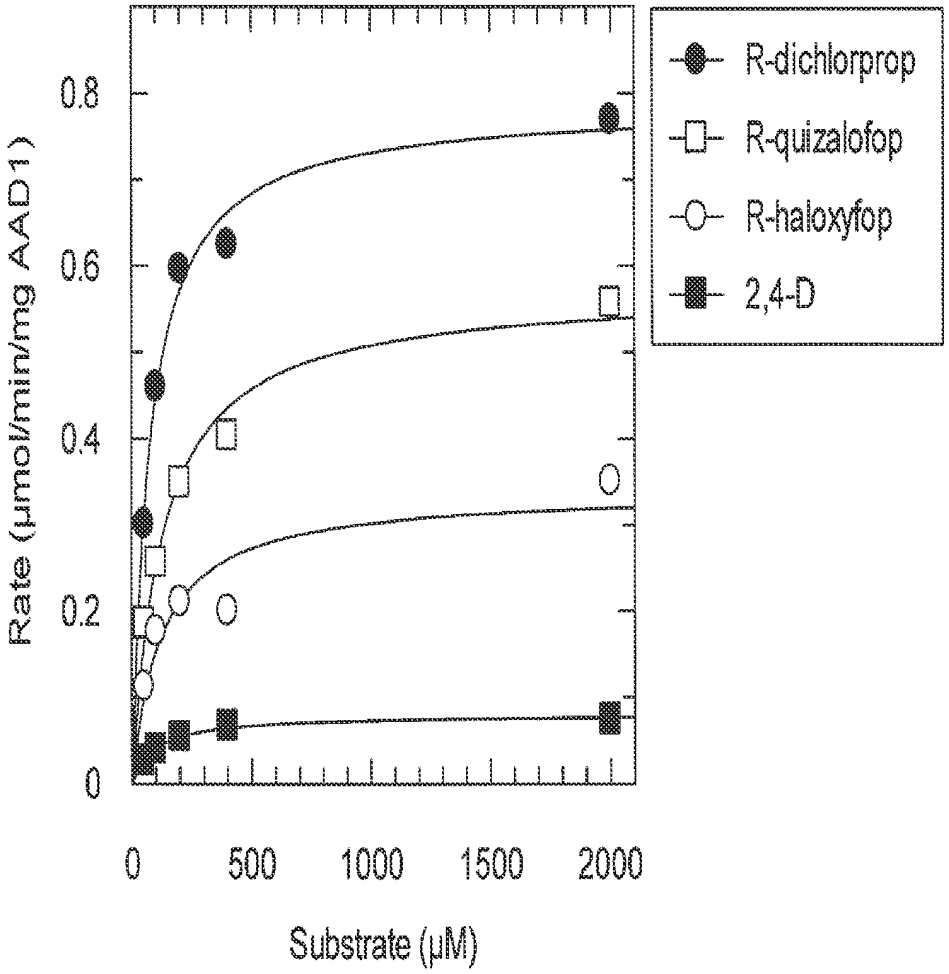

R-dichlorprop
R-quizalofop
R-haloxyfop
2,4-D
Rate (µmol/min/mg AAD1)
0
0.2
0.4
0.6
0.8
0
500
1000
1500
2000
Substrate (µM)

Fig. 7

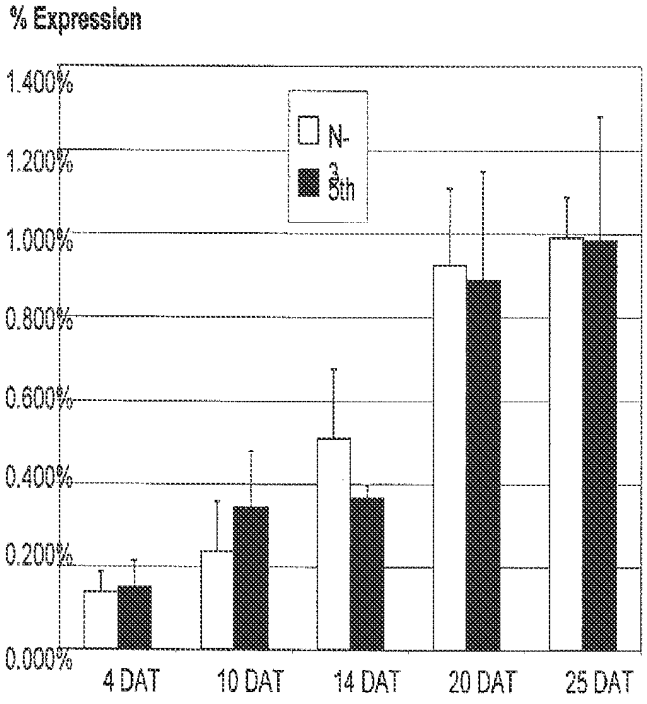

% Expression
1.400%
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8A

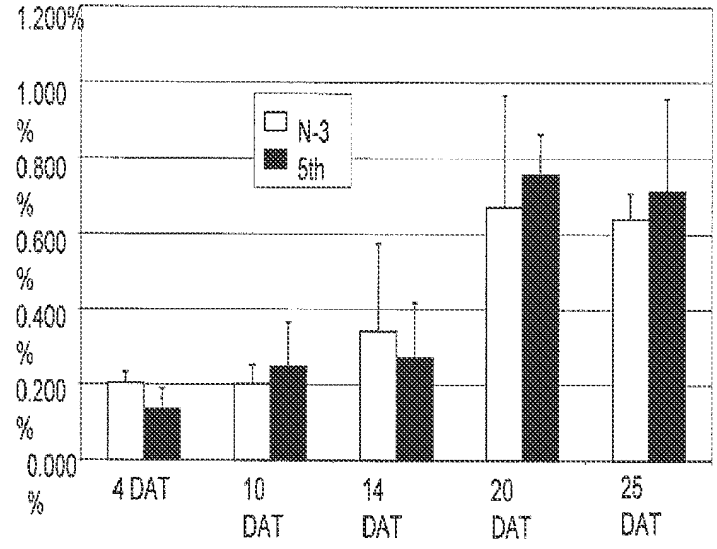

% Expression
1.200%
1.000 %
0.800 %
0.600 %
0.400 %
0.200 %
0.000 %
N-3
5th
4 DAT
10 DAT
14 DAT
20 DAT
25 DAT

Fig. 8B

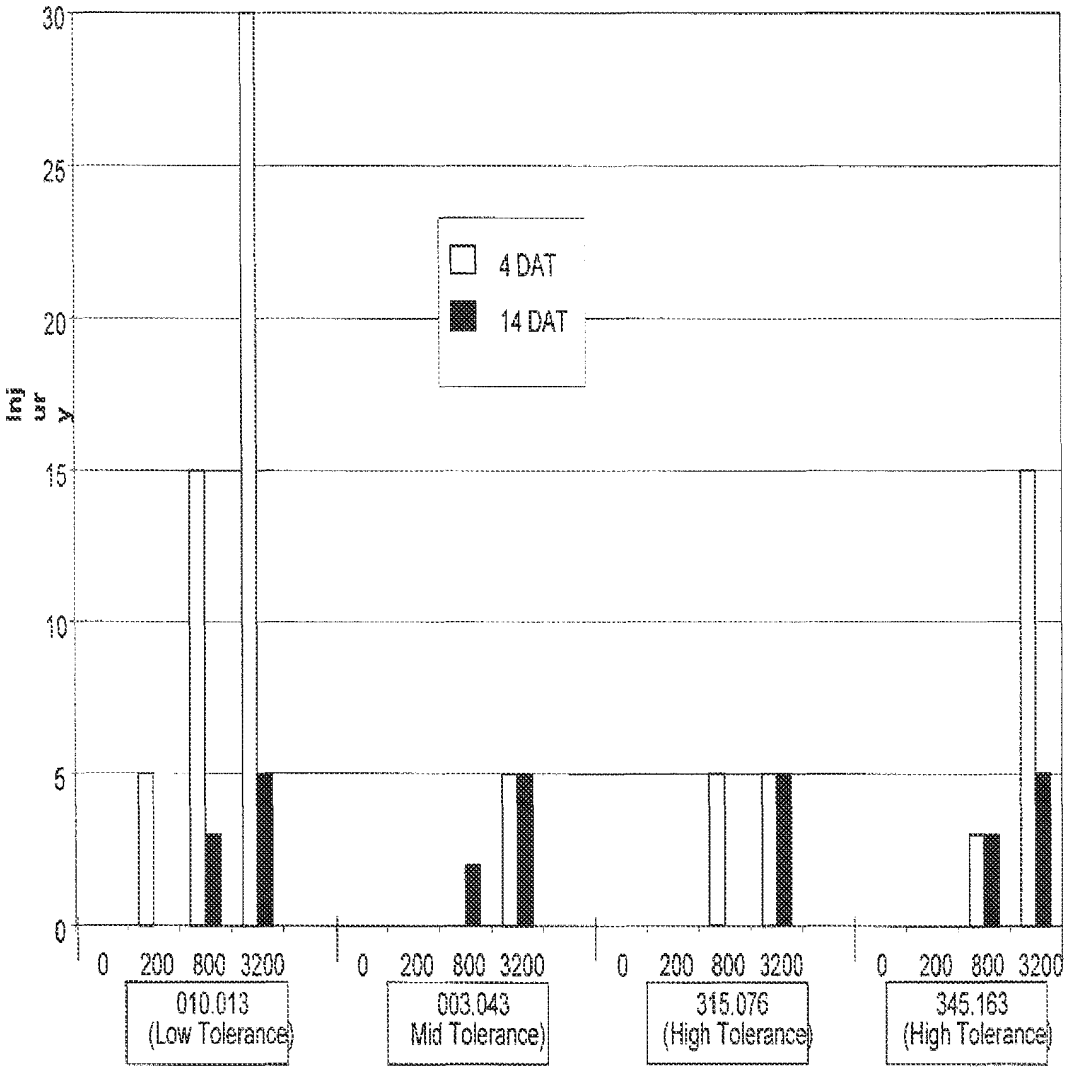

30
25
20
15
10
5
0
4 DAT
14 DAT
0 200 800 3200
010.013
(Low Tolerance)
0 200 800 3200
003.043
Mid Tolerance)
0 200 800 3200
315.076
(High Tolerance)
0 200 800 3200
345.163
(High Tolerance)

Fig. 9A

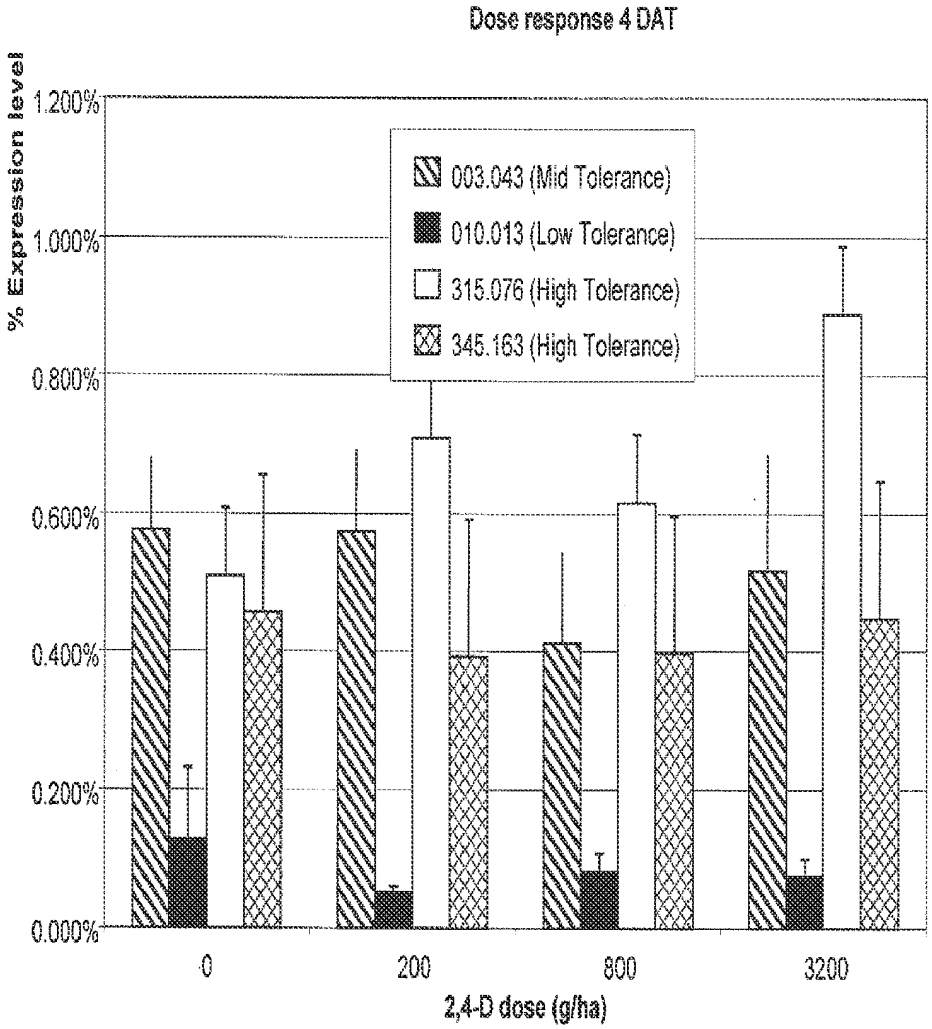

Dose response 4 DAT
% Expression level
1.200%
1.000%
0.800%
0.600%
0.400%
0.200%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/ha)

Fig. 9B

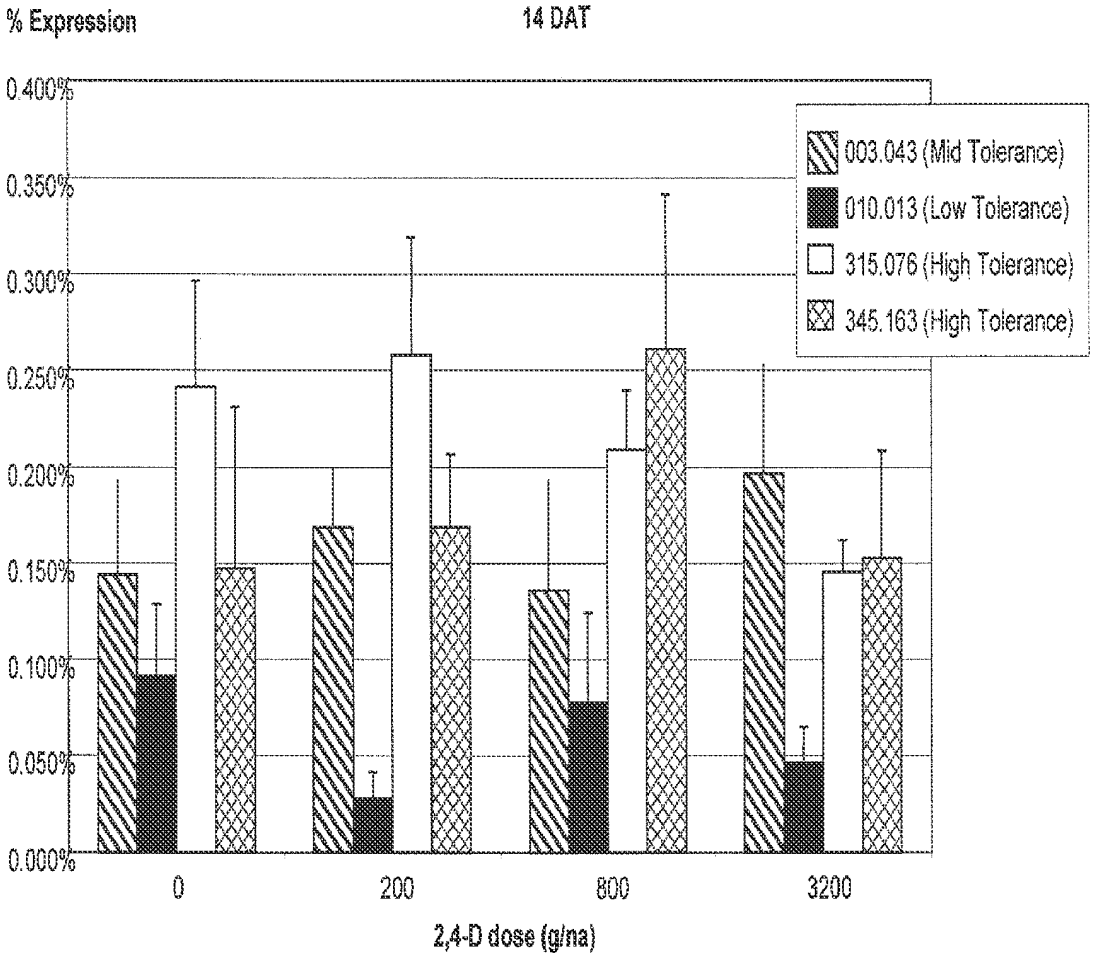

% Expression
14 DAT
0.400%
0.350%
0.300%
0.250%
0.200%
0.150%
0.100%
0.050%
0.000%
003.043 (Mid Tolerance)
010.013 (Low Tolerance)
315.076 (High Tolerance)
345.163 (High Tolerance)
0
200
800
3200
2,4-D dose (g/na)

Fig. 9C

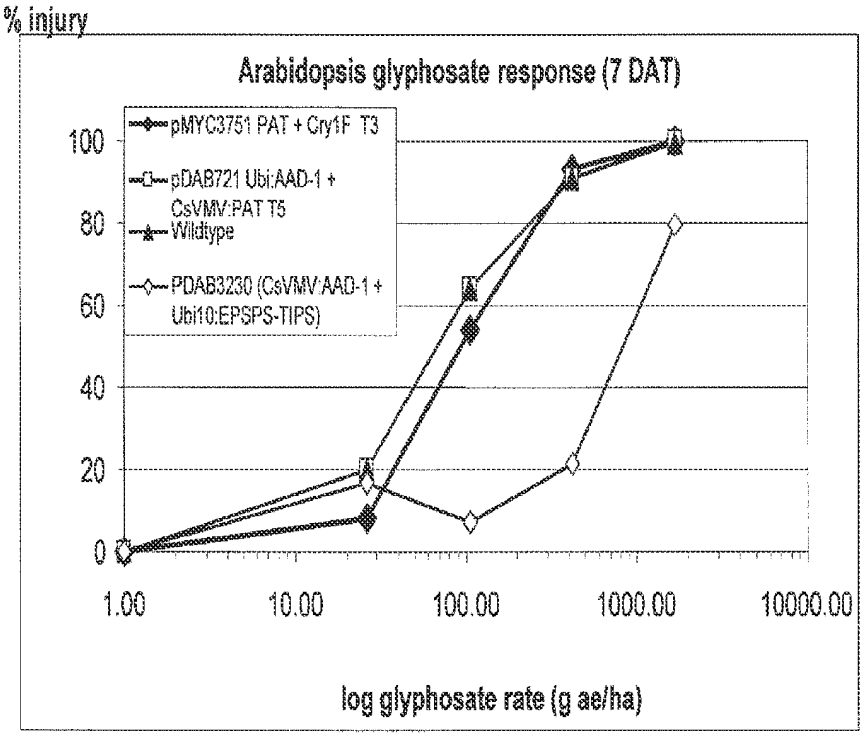

% injury
Arabidopsis glyphosate response (7 DAT)
pMYC3751 PAT + Cry1F T3
pDAB721 Ubi:AAD-1 + CsVMV:PAT T5
Wildtype
PDAB3230 (CsVMV:AAD-1 + Ubi10:EPSPS-TIPS)
100
80
60
40
20
0
1.00
10.00
100.00
1000.00
10000.00
log glyphosate rate (g ae/ha)

Fig. 10

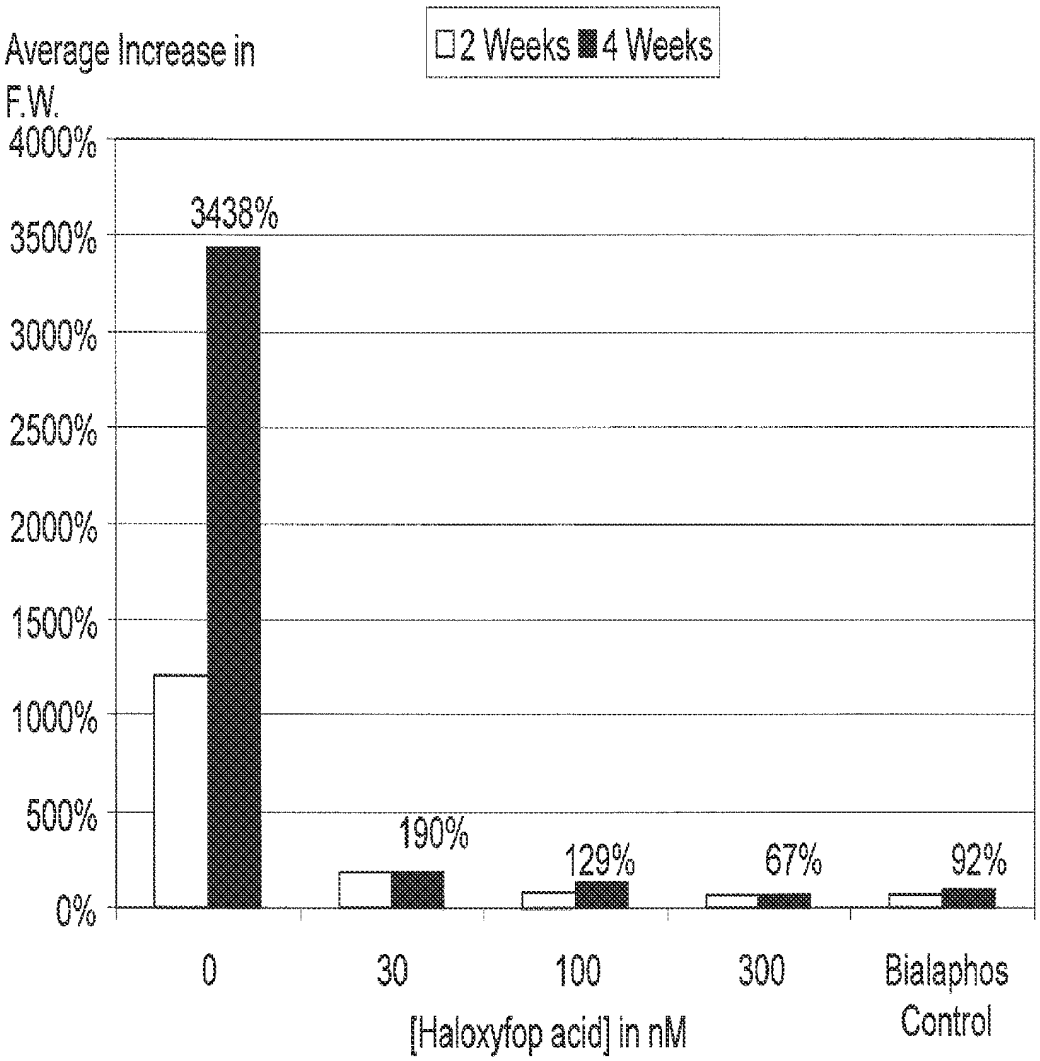

Average Increase in F.W.
2 Weeks
4 Weeks
4000%
3500%
3000%
2500%
2000%
1500%
1000%
500%
0%
3438%
190%
129%
67%
92%
0
30
100
300
Bialaphos Control
[Haloxyfop acid] in nM

Fig. 11

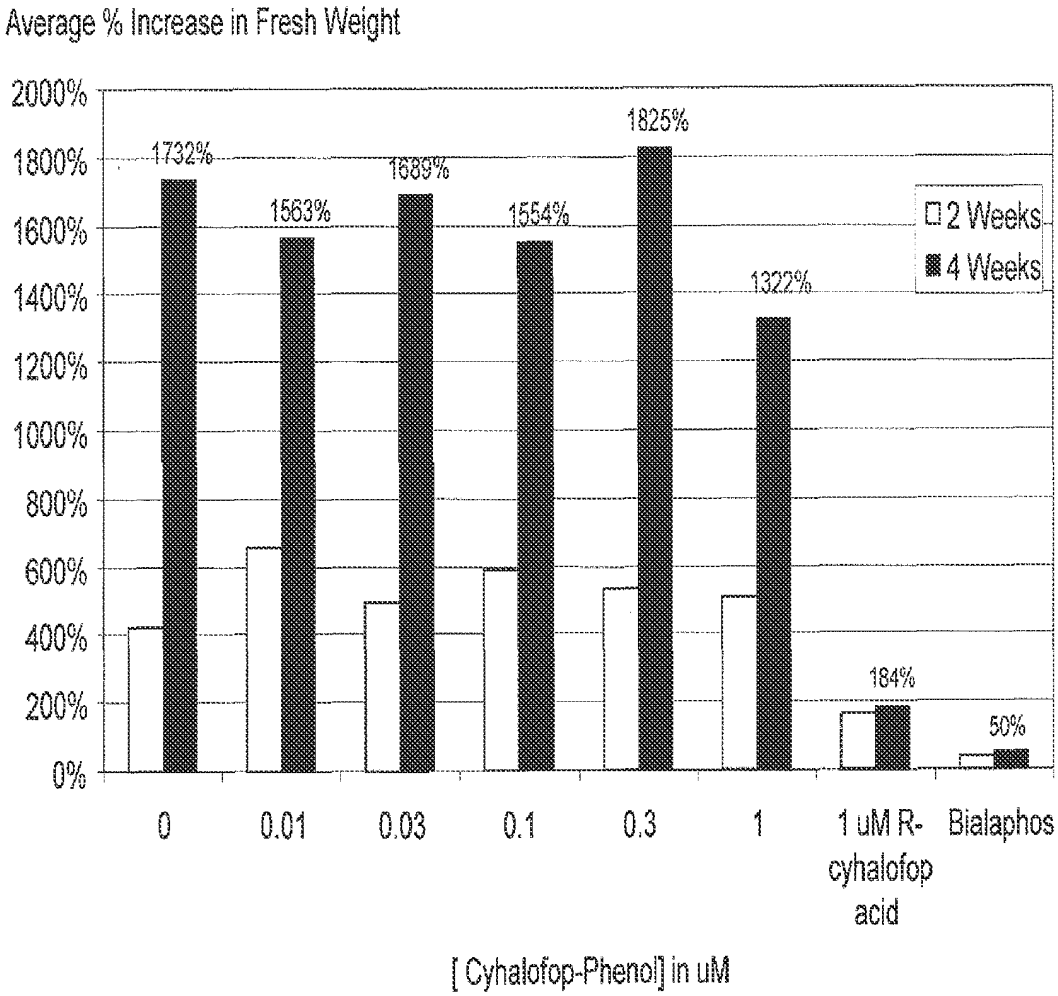

Average % Increase in Fresh Weight
2000%
1800%
1600%
1400%
1200%
1000%
800%
600%
400%
200%
0%
1732%
1563%
1689%
1554%
1825%
1322%
184%
50%
2 Weeks
4 Weeks
0
0.01
0.03
0.1
0.3
1
1 uM R-cyhalofop acid
Bialaphos
[Cyhalofop-Phenol] in uM

Fig. 12

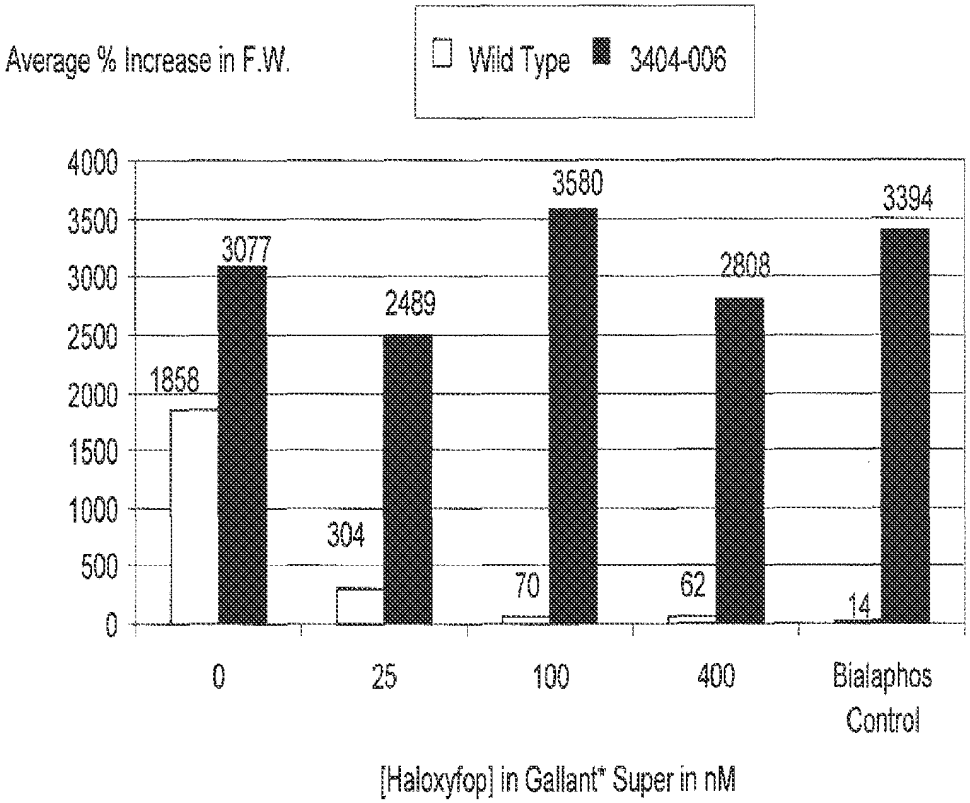

Average % Increase in F.W.
Wild Type
3404-006
4000
3500
3000
2500
2000
1500
1000
500
0
1858
3077
304
2489
70
3580
62
2808
14
3394
0
25
100
400
Bialaphos Control
[Haloxyfop] in Gallant® Super in nM

Fig. 13

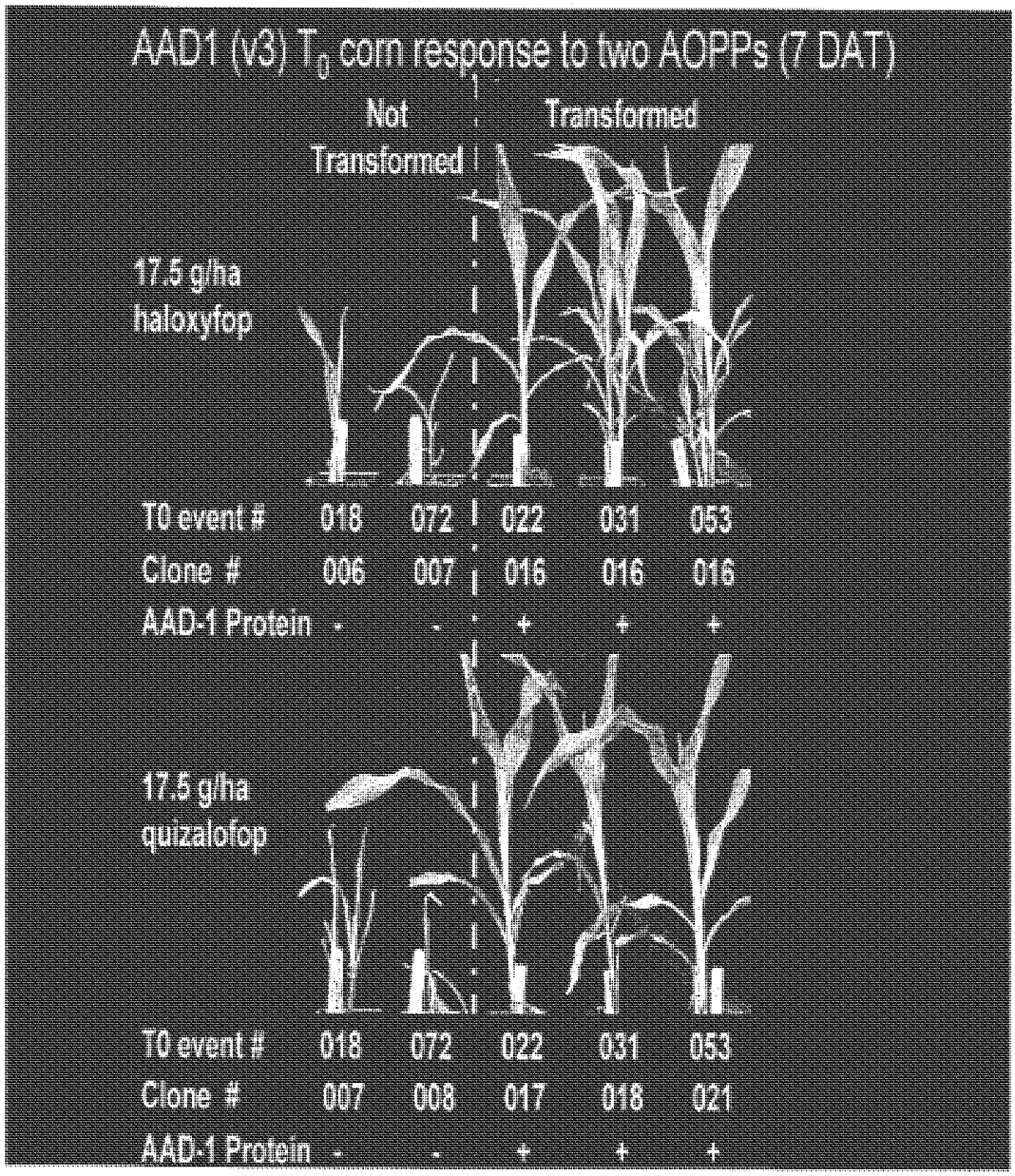

AAD1 (v3) T0 corn response to two AOPPs (7 DAT)
Not Transformed
Transformed
17.5 g/ha haloxyfop
T0 event # 018 072 022 031 053
Clone # 006 007 016 016 016
AAD-1 Protein - - + + +
17.5 g/ha quizalofop
T0 event # 018 072 022 031 053
Clone # 007 008 017 018 021
AAD-1 Protein - - + + +

Fig. 14

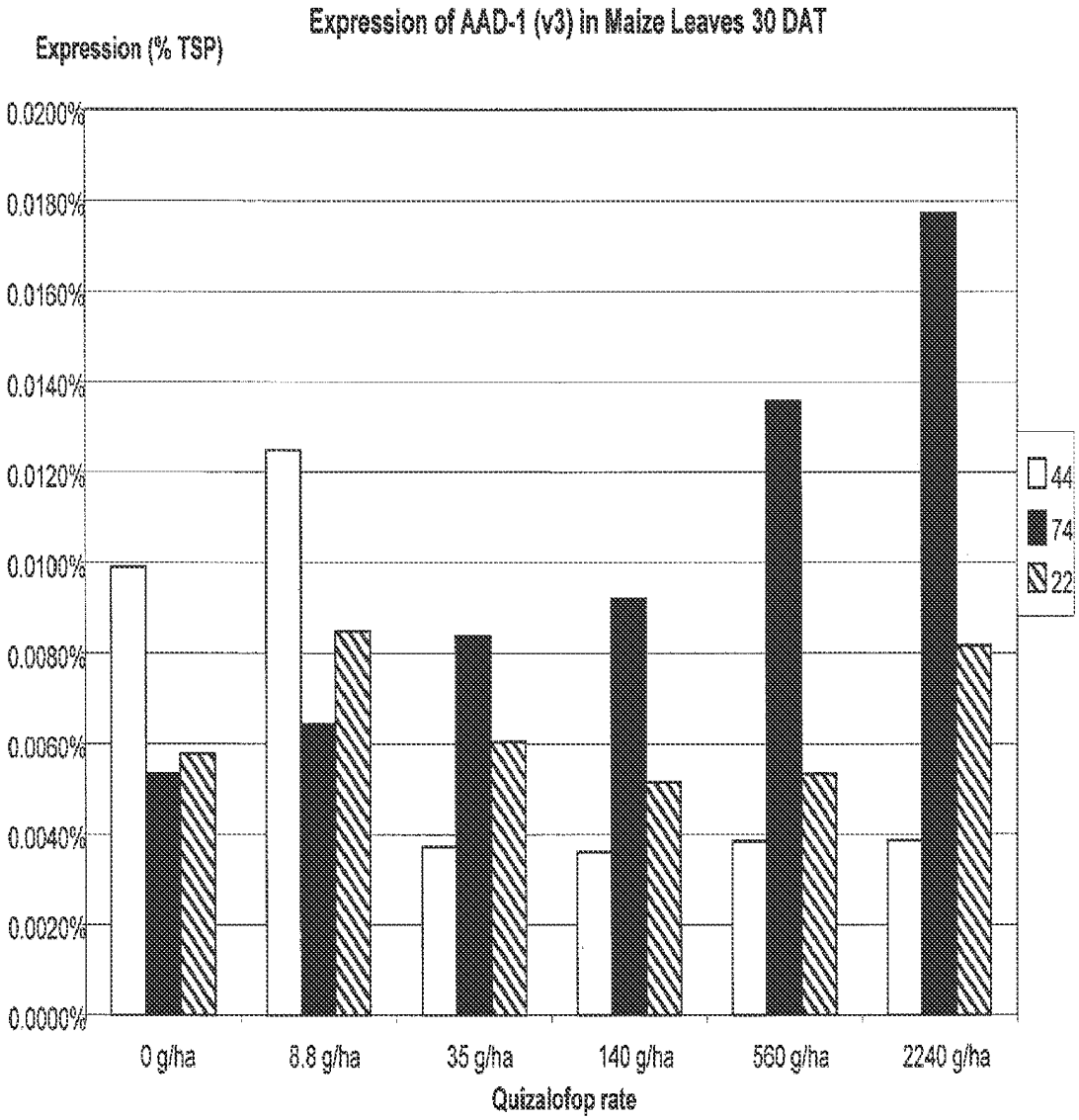

Expression of AAD-1 (v3) in Maize Leaves 30 DAT
Expression (% TSP)
0.0200%
0.0180%
0.0160%
0.0140%
0.0120%
0.0100%
0.0080%
0.0060%
0.0040%
0.0020%
0.0000%
0 g/ha
8.8 g/ha
35 g/ha
140 g/ha
560 g/ha
2240 g/ha
Quizalofop rate
44
74
22

Fig. 15

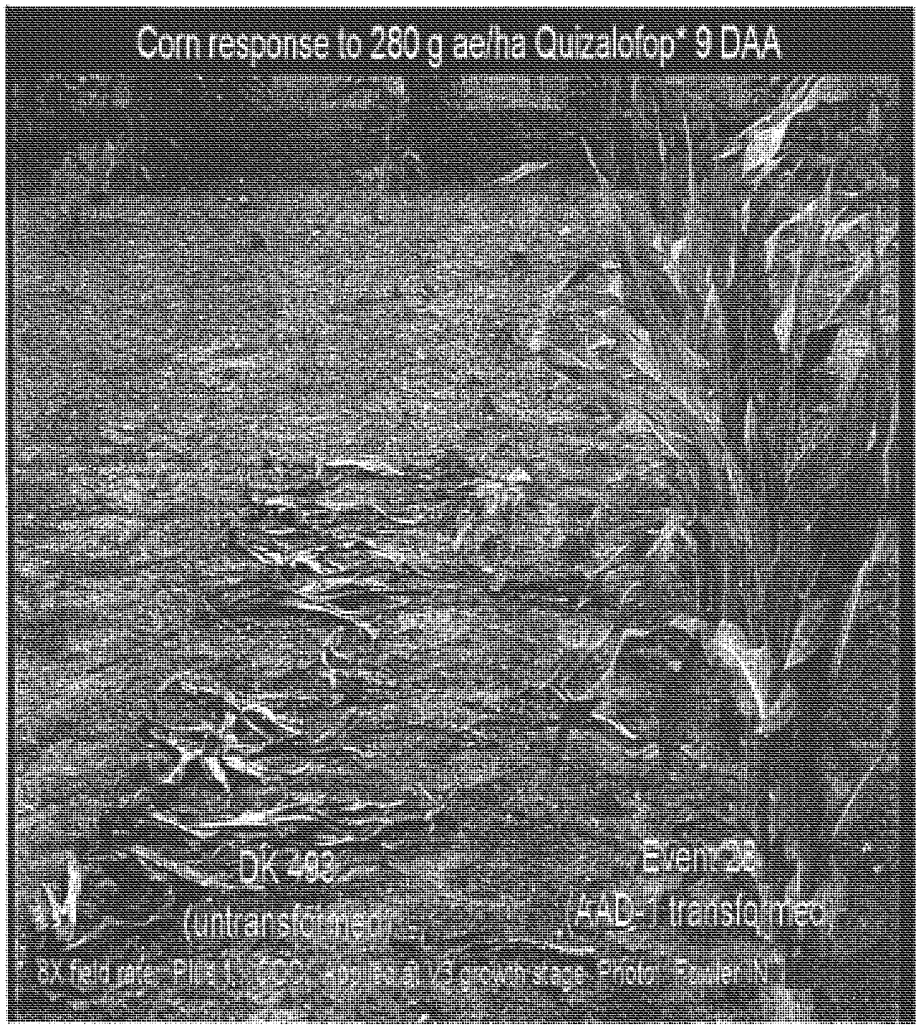

Corn response to 280 g ae/ha Quizalofop* 9 DAA
DK 492
(untransformed)
Event 28
(AAD-1 transformed)

Fig. 16

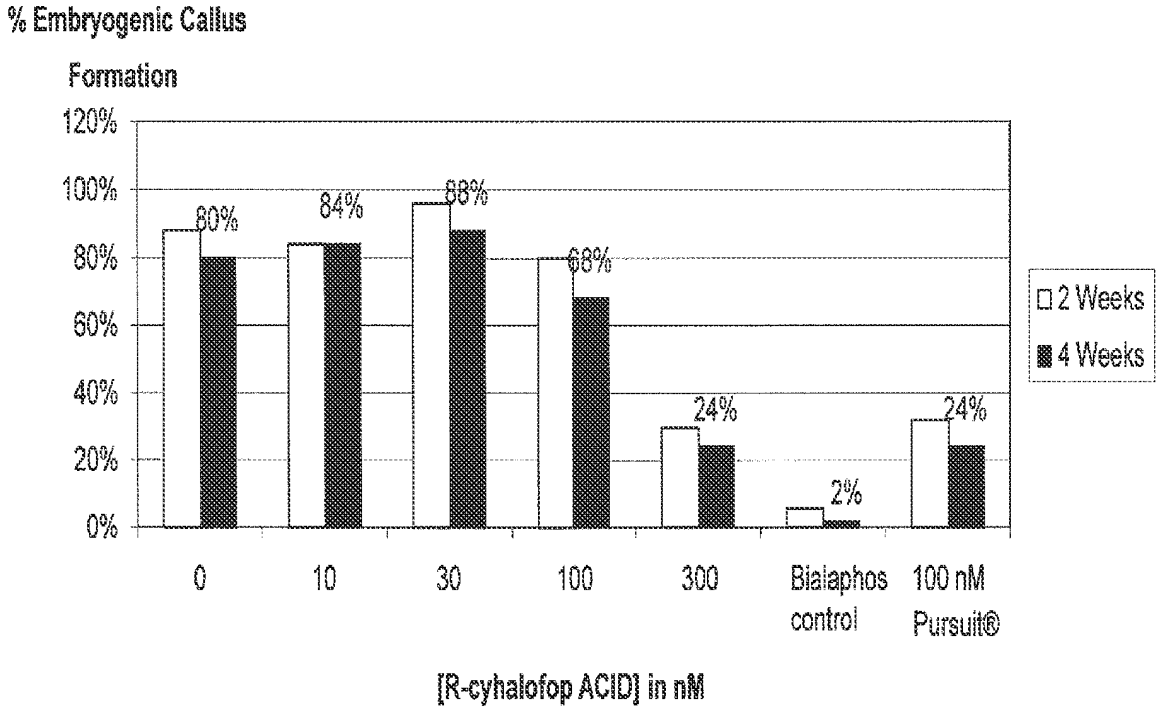
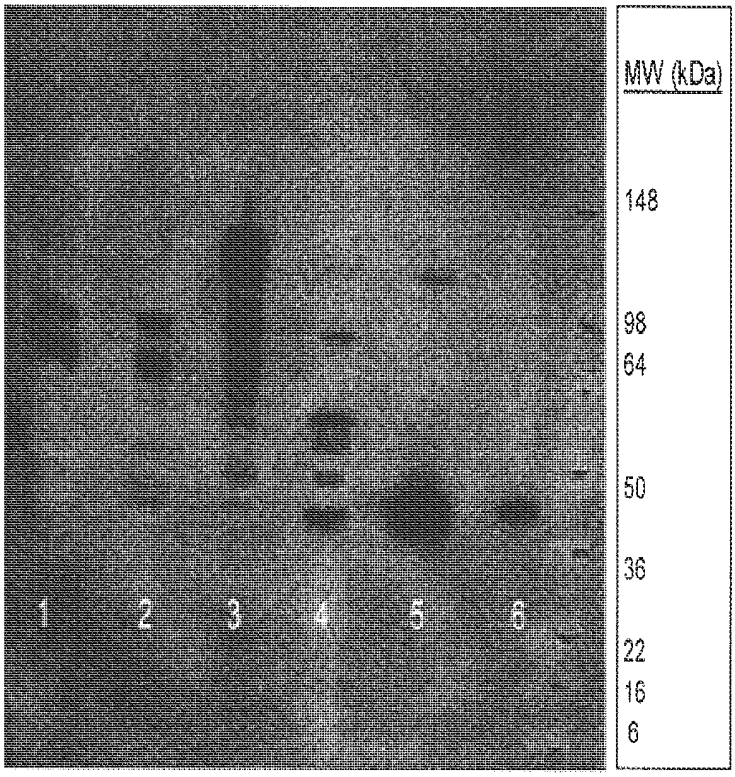

% Embryogenic Callus
Formation
120%
100%
80%
60%
40%
20%
0%
80%
84%
88%
68%
24%
2%
24%
0
10
30
100
300
Bialaphos control
100 nM Pursuit®
2 Weeks
4 Weeks
[R-cyhalofop ACID] in nM

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

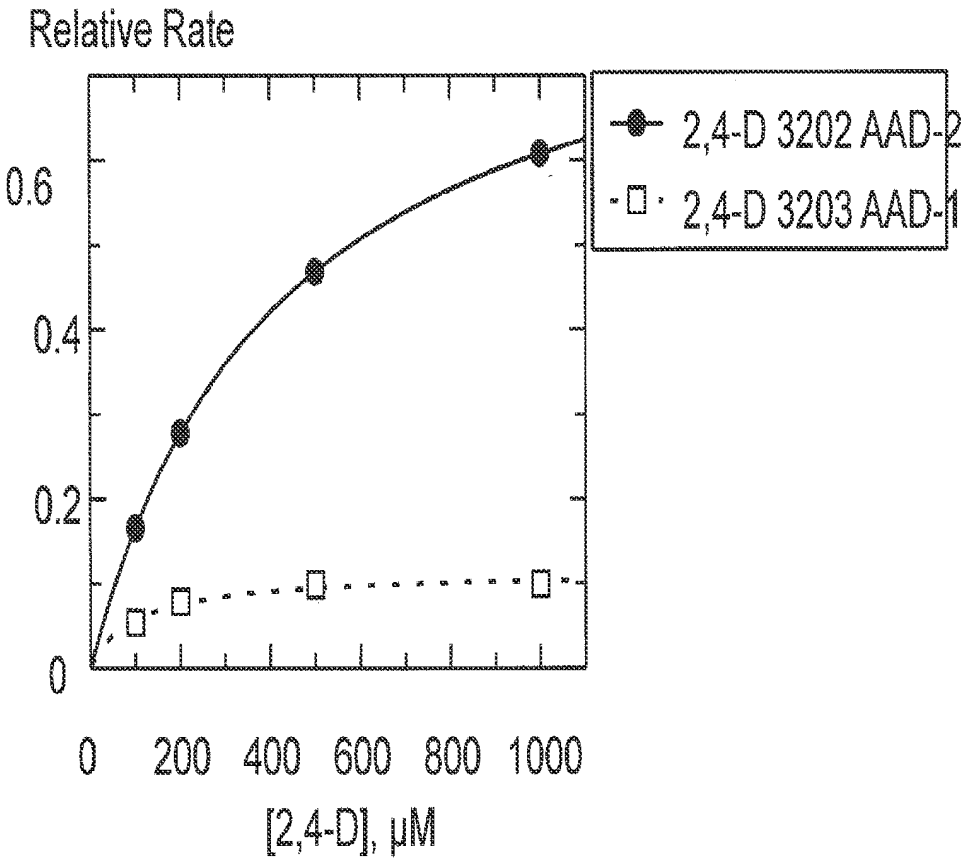

Relative Rate
0.6
0.4
0.2
0
0
200
400
600
800
1000
[2,4-D], µM
2,4-D 3202 AAD-2
2,4-D 3203 AAD-1

Fig. 19

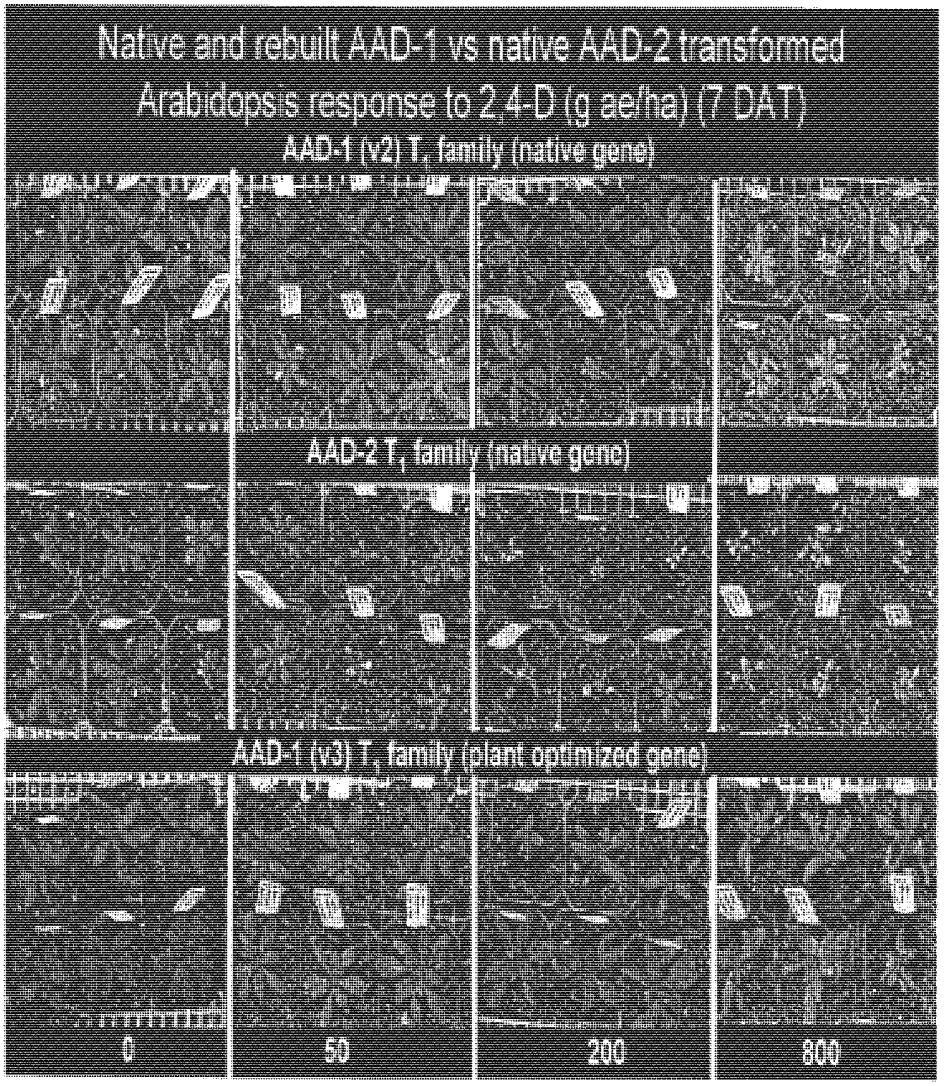

Native and rebuilt AAD-1 vs native AAD-2 transformed
Arabidopsis response to 2,4-D (g ae/ha) (7 DAT)
AAD-1 (v2) T1 family (native gene)
AAD-2 T1 family (native gene)
AAD-1 (v3) T1 family (plant optimized gene)
0
50
200
800

Fig. 20

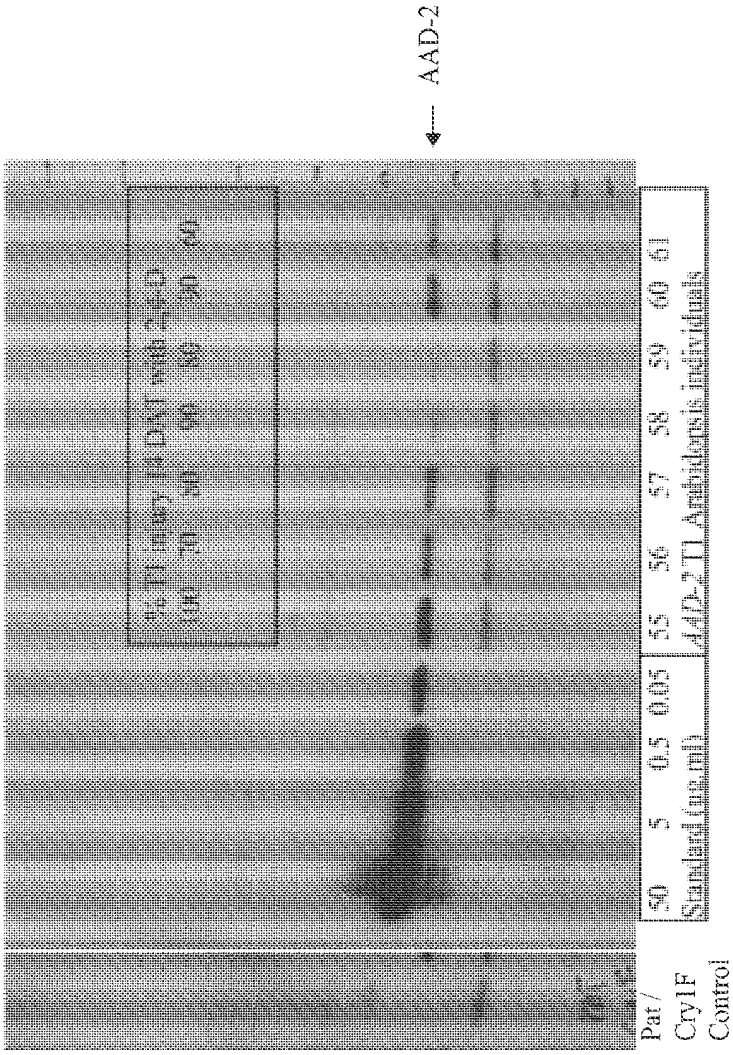

AAD-2
Pat/Cry1F Control
50 5 0.5 0.05 Standard (ug/mL)
55 56 57 58 59 60 61 AAD-2 T1 Antibody individuals

Fig. 21

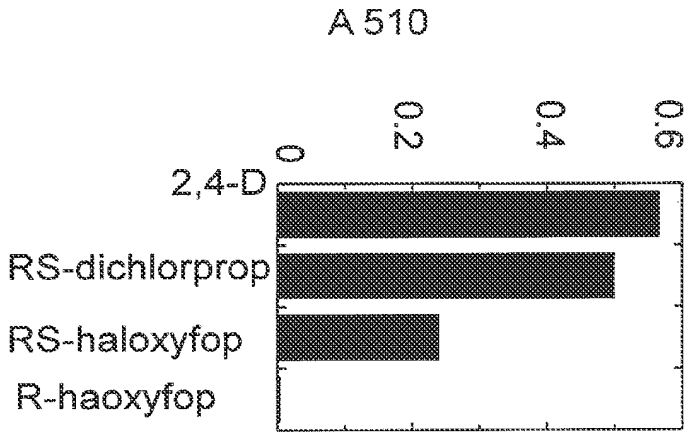


FIG. 22

HERBICIDE RESISTANCE GENES

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation application of U.S. Ser. No. 17/175,966, filed Feb. 15, 2021, now patented as U.S. Pat. No. 11,149,283, which is a continuation application of U.S. Ser. No. 17/143,824, filed Jan. 7, 2021, which is a continuation application of U.S. Ser. No. 15/288,406, filed Oct. 7, 2016, now patented as 10,947,555, which is a continuation application of U.S. Ser. No. 14/820,893, filed Aug. 7, 2015, now patented as U.S. Pat. No. 10,174,337, which is a continuation application of U.S. Ser. No. 12/951,813, filed Nov. 22, 2010, now patented as U.S. Pat. No. 9,127,289, which is a continuation application of U.S. Ser. No. 11/587,893, filed May 22, 2008, now patented as U.S. Pat. No. 7,838,733, which is a national stage entry of PCT/US2005/014737, filed May 2, 2005, which claims the benefit of U.S. Provisional Application Ser. No. 60/567,052, filed Apr. 30, 2004, which are hereby incorporated by reference in their entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, or drawings.

INCORPORATION BY REFERENCES OF MATERIAL SUBMITTED ELECTRONICALLY

Incorporated by reference in its entirety is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: 42 kilobytes ACII (Text) file named "346146_ST25.txt," created on Aug. 16, 2021.

BACKGROUND OF THE INVENTION

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.

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.

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.

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.

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.

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).

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.

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).

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.

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. Pat. 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. Pat. No. 5,608,147).

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.

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).

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.

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

BRIEF SUMMARY OF THE INVENTION

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.

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).

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.

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.

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

FIG. **1** shows a general scheme for dioxygenase cleavage of phenoxy auxin or AOPP herbicides.

FIG. **2** shows loss of herbicidal activity from a 2,4-D solution treated with AAD-1.

FIG. **3** shows loss of herbicidal activity from a haloxyfop solution treated with AAD-1.

FIG. **4** shows anticipated phenols produced from representative herbicides catalyzed by AAD-1.

FIG. **5** shows 2,4-dichlorophenol production by recombinant AAD-1.

FIGS. **6**A and **6**B show phenol production by recombinant AAD-1 from various herbicide substrates.

FIG. **7** shows AAD-1 reaction rate to substrate concentration for four herbicide substrates.

FIGS. **8**A and **8**B 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.

FIGS. **9**A, **9**B, and **9**C 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.

FIG. **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.

FIG. **11** shows dose response of callused maize suspensions to R-haloxyfop.

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

FIG. **13** illustrates dose-response data on one transgenic event, 3404-006, to haloxyfop.

FIG. **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.

FIG. **15** shows three different T2 lineages from 3404 transformations that were pre-screened with Liberty® to remove nulls, which were chosen to compare their tolerance to quizalofop with respect to their AAD-1 expression. Expression was measured at 14 DAT (data not shown) and at 30 DAT.

FIG. **16** shows AAD-1 (v3)-transformed corn tolerant to 8× field rates of quizalofop (Assure II) under field conditions.

FIG. **17** illustrates data from immature maize embryos grown on cyhalofop-containing media.

FIG. **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.

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

FIG. **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.

FIG. **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.

FIG. **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

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

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

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

SEQ ID NO:4 is the nucleic acid sequence of the native AAD-1 gene with internal NotI restriction site removed. This gene is designated AAD-1 (v2). DNA sequencing confirmed that the correct PCR product was generated, but an inadvertent change was made at amino acid #212 from arginine to cysteine.

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.

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).

SEQ ID NO:8 is another PCR primer ("BstEII/Del NotI") that was used with the "3' Sacd" primer.

SEQ ID NO:9 is the native amino acid sequence encoded by the AAD-1 (v1) gene from *Sphingobium herbicidovorans*.

SEQ ID NO:10 is the amino acid sequence encoded by the AAD-1 (v2) DNA sequence of SEQ ID NO:4.

SEQ ID NO:11 is the amino acid sequence encoded by the AMD-1 (v3) plant-optimized DNA sequence of SEQ ID NO:5.

SEQ ID NO:12 is the DNA sequence of the native AMD-2 (v1) gene.

SEQ ID NO:13 is the amino acid sequence of the AAD-2 (v1) protein.

SEQ ID NO:14 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:15 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:16 is the M13 forward primer.

SEQ ID NO:17 is the M13 reverse primer.

SEQ ID NO:18 is a forward primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:19 is a reverse primer used to amplify AAD-2 (v1) DNA for cloning.

SEQ ID NO:20 is the native soybean EPSPS protein.

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).

SEQ ID NO:22 is the soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21.

SEQ ID NO:23 is primer Pat 5-3.

SEQ ID NO:24 is primer Pat 3-3.

SEQ ID NO:25 is forward primer AAD-1 PTU.

SEQ ID NO:26 is reverse primer AAD-1 PTU.

SEQ ID NO:27 is the forward primer for the Coding Region PCR AAD-1.

SEQ ID NO:28 is the reverse primer for the Coding Region PCR AAD-1.

SEQ ID NO:29 is the AAD-2 (v2) nucleotide (plant optimized).

SEQ ID NO:30 is the translated AAD-2 (v2) protein sequence.

SEQ ID NO:31 is the Southern fragment PCR AAD-1 forward primer.

SEQ ID NO:32 is the Southern fragment PCR AAD-1 reverse primer.

DETAILED DESCRIPTION OF THE INVENTION

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.

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

TABLE 1

Commercially available phenoxy auxins.

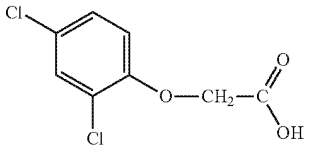
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	

TABLE 1-continued

Commercially available phenoxy auxins.

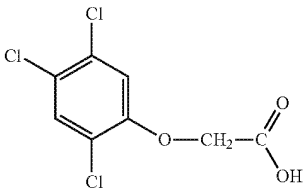
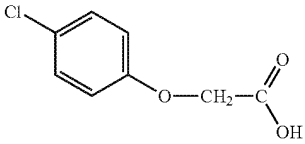
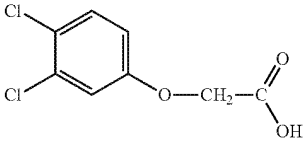
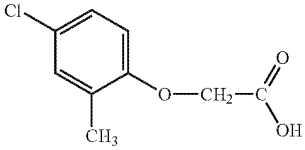
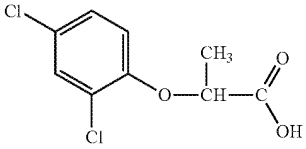
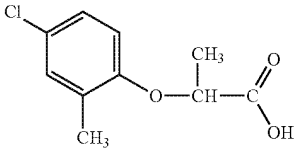
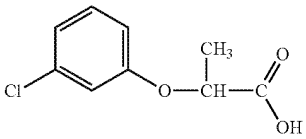
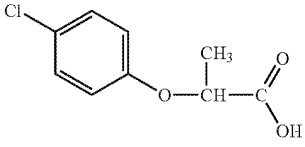
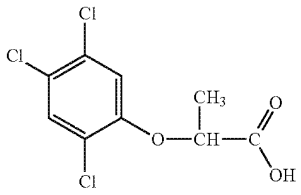
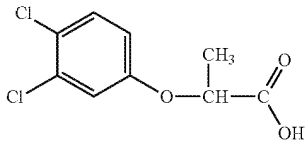
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4,5-T	93-76-5	25-4000	25-4000	
4-CPA	122-88-3	25-4000	25-4000	
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Dichlorprop	120-36-5	22-12000	100-2240	
Mecoprop	7085-19-0	25-4000	250-3360	
Cloprop	101-10-0	25-4000	25-4000	
4-CPP	3307-39-9	25-4000	25-4000	

TABLE 1-continued

Commercially available phenoxy auxins.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Fenoprop	93-72-1	25-4000	25-4000	
3,4-DP	3307-41-3	25-4000	25-4000	

An additional benefit of the AAD-1 gene is its unprecedented ability to concomitantly degrade a host of commercial and non-commercial graminicidal compounds of the general class aryloxyphenoxypropionates (AOPPs). See Table 2. This attribute may allow the use of any of a number of AOPP compounds in transgenic crops containing AAD-1, where tolerance in those crops had not previously warranted use in those crops. These will most commonly include grass crops such as corn, rice, wheat, barley, rye, oats, sorghum, warm and cool-season turf species, grass pasture species, and many others, but could also include dicot crops where AOPP tolerance (naturally present in most dicots) is not at commercially acceptable levels to allow AOPP use in said dicot crop.

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

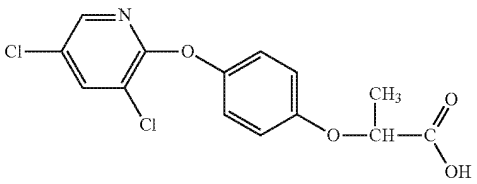
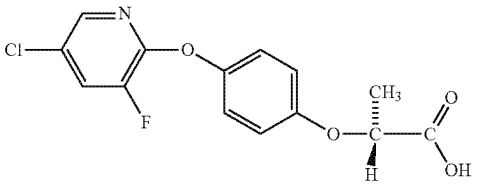
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Chlorazifop	72492-94-7	10-2000	10-2000	
Clodinafop	105512-06-9	10-2000	20-200	

TABLE 2-continued

AOPP graminicidal compounds listed by accepted common names

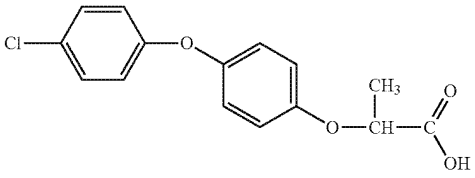
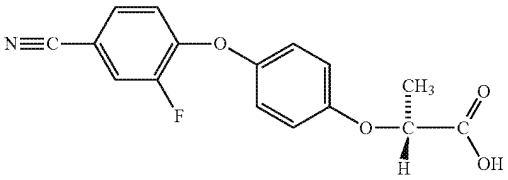
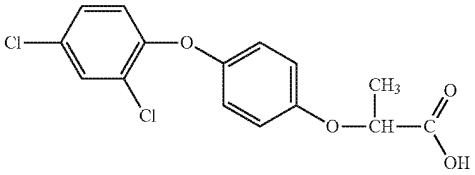
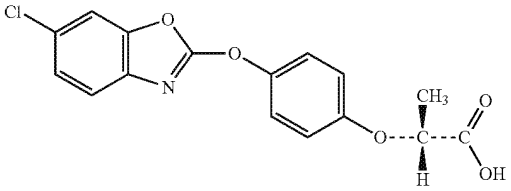
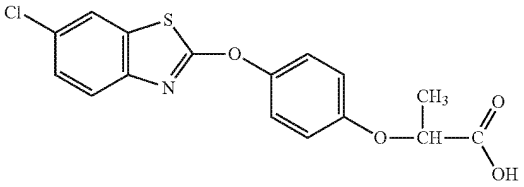
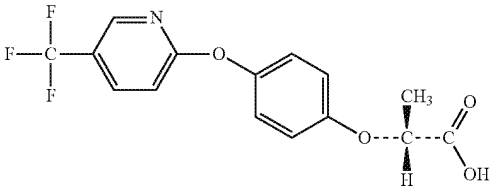
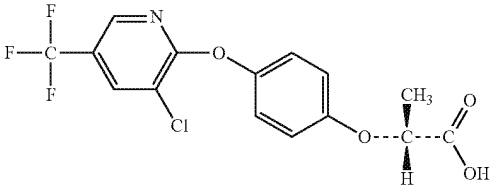
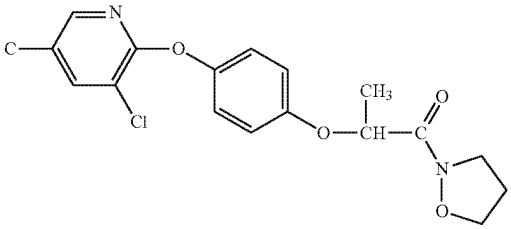
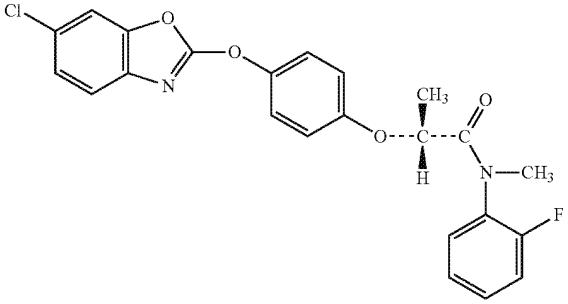
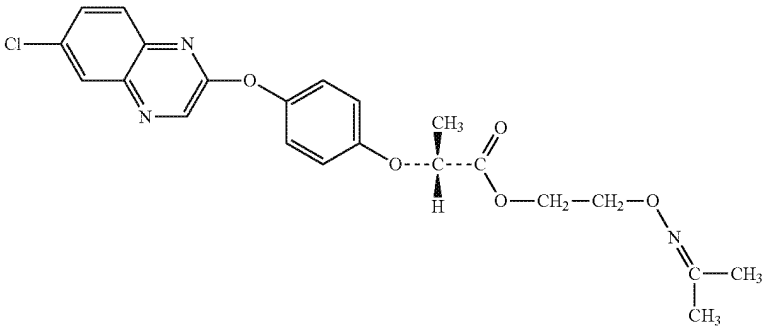
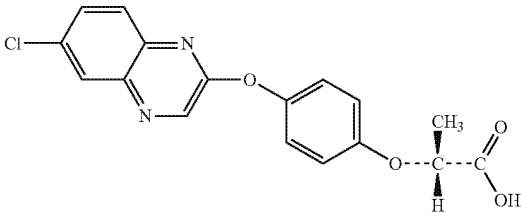
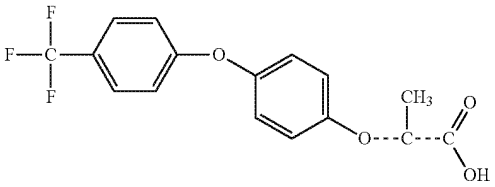
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
Clofop	59621-49-7	10-2000	10-2000	
Cyhalofop	122008-85-9	10-2000	105-560	
Diclofop	71283-65-3	10-2000	280-2000	
Fenoxaprop	66441-23-4	10-2000	20-200	
Fenthiaprop	95721-12-3	10-2000	10-2000	
Fluazifop	69335-91-7	10-2000	25-420	
Haloxyfop	69806-40-2	10-2000	20-600	

TABLE 2-continued

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
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	

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

for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several AOPP graminicidal compounds may be applied to plants expressing AAD-1 with reduced risk of injury from said herbicides. The rate for each AOPP may range from 10 to 2000 g ae/ha, and more typically from 20-500 g ae/ha for the control of a broad spectrum of monocot weeds. Combinations of these different chemistry classes and herbicides with different modes of action and spectra in the same field (either sequentially or in tank mix combination) shall provide control of most potential weeds for which herbicidal control is desired.

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).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-1 stack could range from 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from 25-4000 g ae/ha; and AOPP herbicide(s) (one or more) could be applied from 10-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

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.

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.

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).

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.

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.

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. As disclosed herein a peptide of SEQ ID NO: 9 is provided that is capable of degrading 2,4-D, and AOPP herbicides. Mapping the α-ketoglutarate-dependent dioxygenase motif disclosed in the previous paragraph to the sequence of SEQ ID NO: 9 reveals the AAD-1 motif of $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9:

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids.

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.

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.

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.

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.

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.

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. Pat. 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.

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).

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).

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 1 mM 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).

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 (10×) purified AAD-1 (v1) per 0.16 ml assay.

TABLE 3

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

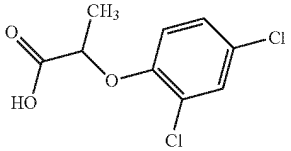
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	117613	(R,S)-dichlorprop	0.566	2.594

TABLE 3-continued

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

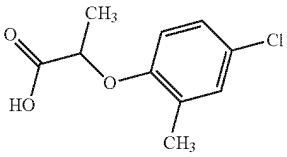
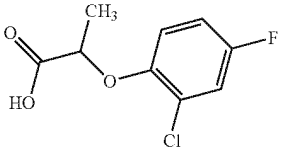
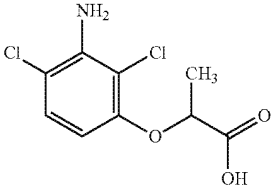
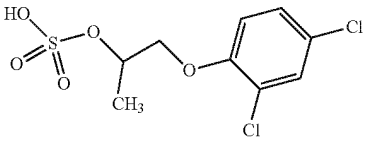
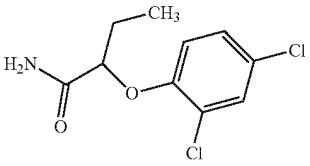
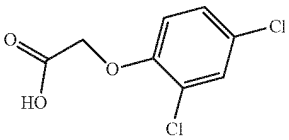
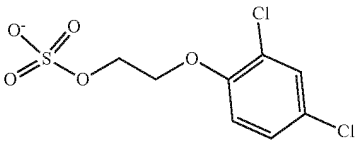
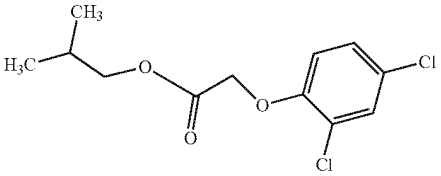
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	188874	(R,S)-mecoprop	0.341	2.085
	83293	(R,S)-2-chloro, 4-fluorophenoxy-proprionate	0.304	2.358
	11113675	(R,S)-3-aminodichlorpop	0.228	2.676
Na^+ 	188476		0.077	0.687
	192132		0.064	0.204
	195517	2,4-D	0.034	0.383
Na^+ 	398166	sesone	0.02	0.177
	190252		0.008	0.211

TABLE 3-continued

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

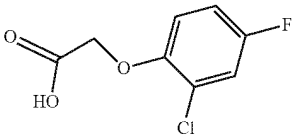
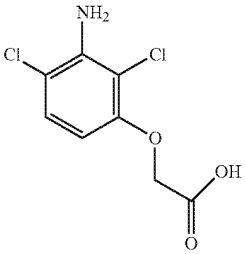
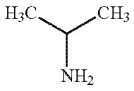
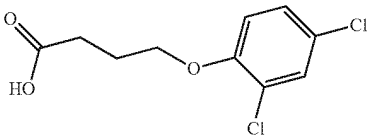
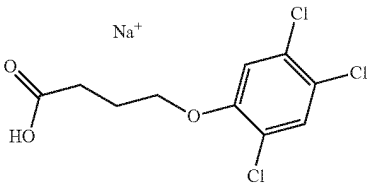
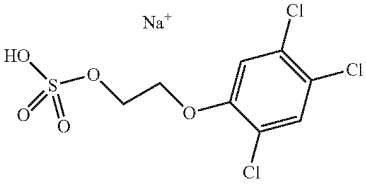
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	124988		0.007	0.058
	11263526		0.004	0.069
	178577		0.003	0.021
				
	178587		0.003	0.02
	188527		0.003	0.036

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 (10×) 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.

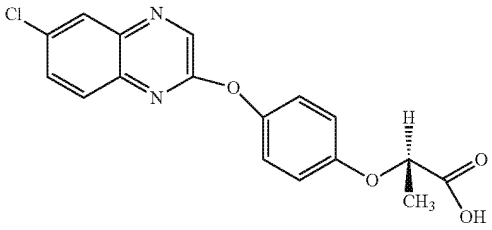
STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	18706	(R)-quizalofop	0.43	2.1

TABLE 4-continued

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

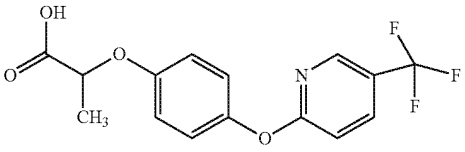
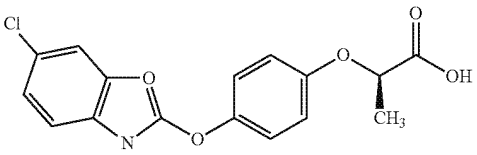
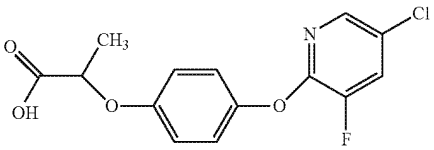
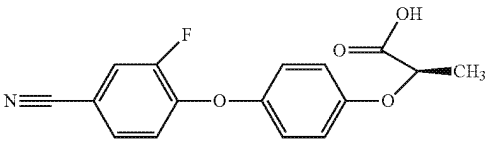
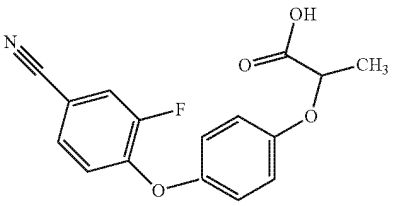
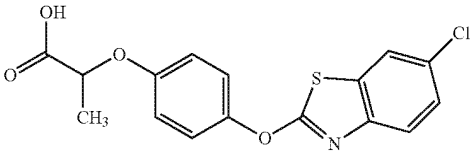
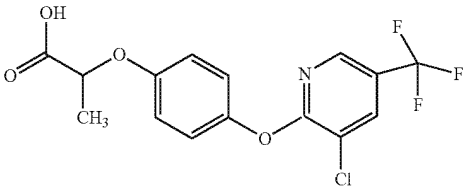
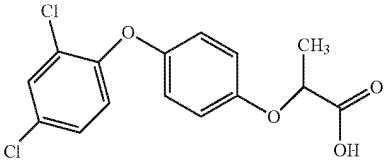
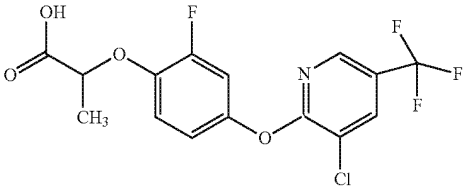
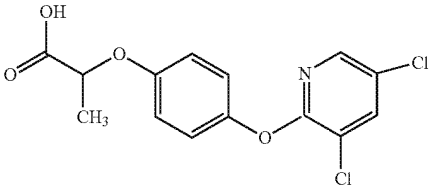
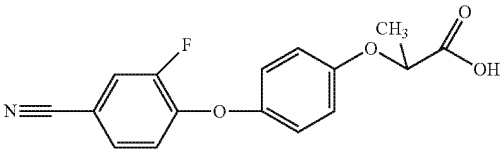
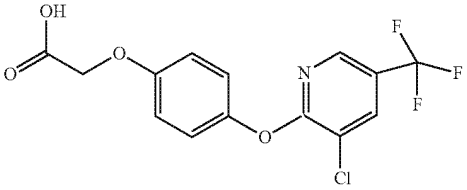
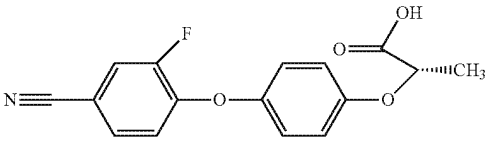
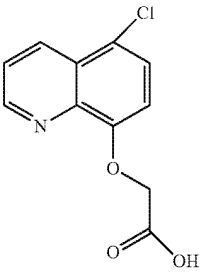
STRUCTURE		Registry ID	Compound	AAD1	AAD1 (10X)
		67131	(R,S)-fluazifop	0.427	2.17
	Chiral	11044492	(R)-fenoxaprop	0.408	0.597
		34697	(R,S)-clodinofop	0.295	1.98
		14603	(R)-cyhalofop	0.222	1.989
		14623	(R,S)-cyhalofop	0.215	1.815
		62942	(R,S)-fenthiaprop	0.199	1.055
		66905	haloxyfop	0.172	1.63
		460511	(R,S)-diclofop	0.155	1.663

TABLE 4-continued

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

STRUCTURE	Registry ID	Compound	AAD1	AAD1 (10X)
	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	Cloquinotocet	0	0.001

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 pro-

teins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.).

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, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2×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 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×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 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×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.

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.

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.

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 6×SSPE, 5×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):

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

Washes can typically be carried out as follows:

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

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

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

$$Tm\ (° C.) = 2\ (\text{number } T/A \text{ base pairs}) + 4\ (\text{number } G/C \text{ base pairs})$$

(Suggs et al., 1981).

Washes can typically be out as follows:

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

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

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 2×SSPE, room temperature

Low: 1 or 2×SSPE, 42° C.

Moderate: 0.2× or 1×SSPE, 65° C.

High: 0.1×SSPE, 65° C.

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.

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. Pat. 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.

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.

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.

In addition, U.S. Pat. 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.

"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.

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.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

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.

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.

Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits. For example, in one embodiment an amino acid sequence is provided having at least 85%, but less than 95% sequence identity with SEQ ID NO: 9; and an AAD-1 motif having the general formula of: $HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$, wherein

X_{112} represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids.

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.

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

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.

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."

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."

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.

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.

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.

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.

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.

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. Pat. No. 5,135,867.

Vectors comprising an AAD-1 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley et al. (1986); and An et al. (1985).

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.

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.

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.

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.

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. No. 4,945,050 to Cornell and U.S. Pat. No. 5,141,131 to Dow-Elanco, 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; U.S. Pat. No. 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 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. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

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.

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

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.

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.

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.

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.

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, 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.

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.

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. No. 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.

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.

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

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.

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.

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.

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' TCTAGAAGGAGATATACC 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' CTCGAGTTA CTA GCG CGC CGG GCG CAC GCC ACC GAC CG 3' [(SEQ ID NO:2)(added extra stop codon and Xho I site)].

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 ~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.

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).

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.

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.

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.

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).

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.

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.

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) 250×4.6 mm (Phenomenex cat. #OOG-4253-EO), run at 50% ACN+0.05% TFA: 50% H_2O+0.05% TFA to 100% ACN+0.05% TFA over 5 min.

2.2—Plate Test Bioassays for Herbicide Degradation.

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.

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.

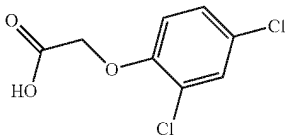
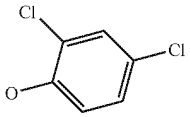
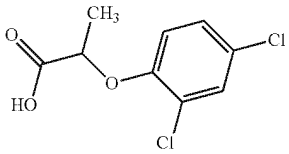
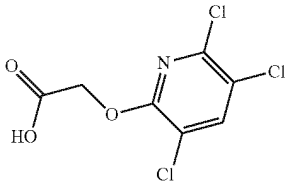
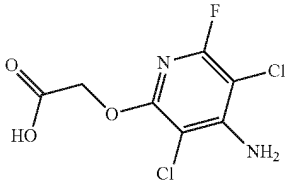
Twenty to thirty sterilized seeds for each test species used were added onto a sterile, solidified agar Plate Test Medium (PTM) [2.5 mM KNO_3, 2.5 mM KH_2PO_4, 50 mM $FeSO_4$, 10 mM NaEDTA (pH 8.0), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4.2H_2O$, 10 μM NaCl, 10 nM $CoCl_2.H_2O$, 0.8% (w/v) sucrose, 0.4% agarose (w/v)] for bioassay in 60×15-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.

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.

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

TABLE 6

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

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

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

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

TABLE 7

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

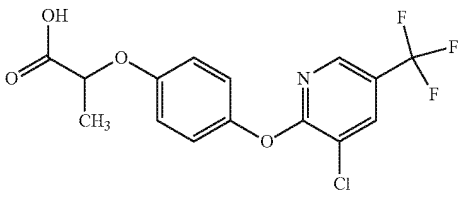
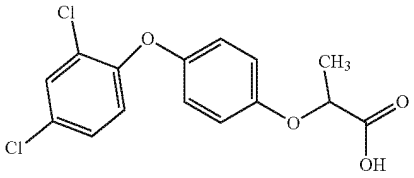
Chemical tested	GR50 (nM) Chemical alone	Chemical + Blank Vector*	Chemical + AAD-1 v1	GR50 ratio**	Structure
Haloxyfop-RS	28	21	520	25	

TABLE 7-continued

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

	GR50 (nM)				
Chemical tested	Chemical alone	Chemical + Blank Vector*	Chemical + AAD-1 v1	GR50 ratio**	Structure
Diclofop-RS	nd	20	130	7	

*Blank vector represents of cell lysate treatment where *E. coli* pET vector had 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.

2.3—HPLC Results.

From the literature, it was known that dioxygenase enzymes in this class require α-ketoglutarate as a co-substrate (for a general scheme, see FIG. **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.

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 (FIG. **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.

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).

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 (FIG. **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.

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 (FIG. **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.

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 (FIG. **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

3.1—AAD-1 (v1) Assay.

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 FIG. **4**). The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 μM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 μM $NH_4(FeSO_4)_2$, 200 μM sodium ascorbate. The phenols derived from haloxyfop and cyhalofop had equivalent color

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

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.

3.2—Extraction.

Activity of recombinant AAD-1 (v1) expressed in *Escherichia coli*. *E. coli* cell pellets were resuspended in 0.1 M Tris, pH 7.4+1 mg/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.

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 FIG. **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.

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. FIG. **6**A 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.

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 FIG. **6**B. 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—Additional Substrates of AAD-1 (v1).

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).

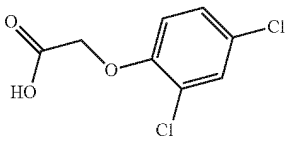
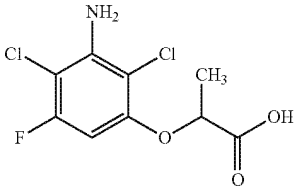
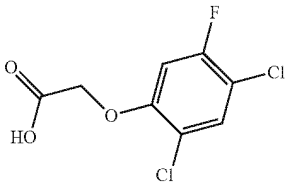
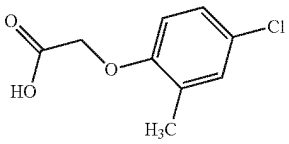
Table 4 shows data for a variety of AOPP graminicide herbicides as AAD1 substrates, and also the safener cloquintocet. All the commercial AOPP herbicides tested were effectively cleaved by AAD-1 (v1). This is an unexpected discovery and greatly increases the potential utility of this enzyme for conferring resistance to a wide variety of graminicidal herbicides in transgenic uses, in addition to auxins. AAD-1 (v1) had the highest activity on quizalofop (76% of the dichlorprop rate) and lowest activity on cyhalofop (27% of the quizalofop rate, 21% of the dichlorprop rate). The aryloxyacetate analog of haloxyfop (X043865) was cleaved very slowly with only a small increase in A_{510} using the higher (10 μg) amount of enzyme. This is consistent with higher activity of AAD-1 (v1) seen on phenoxypropionates relative to auxin phenoxyacetates. Minimal activity was detected on (S)-cyhalofop indicating that AAD-1 (v1) has a significant preference for the R enantiomers of aryloxypropionate substrates. Similarly, no activity was noted against the quinolinoxyacetate safener, cloquintocet, which is consistent with the observation that AAD-1 (v1) prefers aryloxypropionate substrates over phenoxy auxins.

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-aminodichlorprop, 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.

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

TABLE 8

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

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

4.2—Kinetic Characterization.

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 FIG. 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).

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

TABLE 9

Kinetic Constants for AAD-1 (v1) Substrates.

Substrate	K_m (μM) ± SE	V_{max} (μmol min^{-1} mg^{-1} AAD1) ± SE	kcat (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

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.

4.3—Additional Substrates for AAD-1 (v1).

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

5.1—Background.

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.

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.

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 MacVector™ program (Accelerys, San Diego, Calif.). 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.aNumber of genes in class given in parentheses.
.sup.bStandard deviations given in parentheses.
.sup.cCombined groups mean ignored in mean calculation

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.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 11. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

TABLE 11

Preferred amino acid codons for proteins expressed in maize

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

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.

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. Pat. No. 5,380,831.

5.2—Rebuild Analysis.

Extensive analysis of the 888 base pairs (bp) of the DNA sequence of the native AAD-1 (v1) coding region (SEQ ID NO:3) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. To improve production of the recombinant protein in monocots as well as dicots, a "plant-optimized" DNA sequence (SEQ ID NO:5) was developed that encodes SEQ ID NO:11, which is the same as the native SEQ ID NO:9 except for the addition of an alanine residue at the second position. The additional alanine codon (GCT; underlined in SEQ ID NO:5) was included to encode an Nco I site (CCATGG) spanning the ATG start codon, to enable subsequent cloning operations. The proteins encoded by the native (v1) and plant-optimized (v3) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native (v1) and plant-optimized (v3) DNA sequences of the coding regions are only 77.7% identical. A sequence alignment was made of the native and plant-optimized DNAs, and Table 12 shows the differences in codon compositions of the native and plant-optimized sequences.

TABLE 12

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

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

5.3—Completion of Binary Vectors.

5.3.1—Rebuilt AAD-1 Wv3). 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).

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).

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.

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.

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).

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.

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*.

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.

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the "kazusa.or.jp/codon" World Wide Web site. Those data were reformatted as displayed in Table 13. Columns D and H of Table 13 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 13. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

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

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

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 Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H 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

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.

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 Tex.).

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, Calif. 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.

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

TABLE 14

Binary constructs used in transformation of various plant species.

pDAB #	pDAS #	Species* Trans-formed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
721		A, T, Ct, S, Ca	AAD1 v3	AtUbi10	NtOsm	----	----	----	Erythro-mycin	----	pat	CsVMV	Agro binary
3230		A	EPSPS	AtUbi10	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	AAD1 v3	CsVMV	Agro binary
3289		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	sacB	HptII	AtUbi3	Agro binary
3291		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	—	HpII	AtUbi3	Agro binary
3295		S	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	pat	AtUbi10	Aerosol beam
3297	1270	A, T	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	----	----	Spectino-mycin	----	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	—	—	Spectino-mycin	—	AHAS v3	OsAct1	Agro Super-binary
3757		Ca	AAD1 v3	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	----	pat	AtUbi11	Agro binary
3705		A	AAD2 v2	AtUbi10	NtOsm	RB7 Mar v2	----	----	Erythro-mycin	----	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

6.1—*Arabidopsis thaliana* Growth Conditions.

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).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) 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.

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^2 sec under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

6.2—*Agrobacterium* Transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/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, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

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: 2.4 kV, Pulse length: 5 msec.

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

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, Wis.) per manufacturer's instructions at 0.5× 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.

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. 8700× g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing: ½× Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044 μM 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.

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 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), 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.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) 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).

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.

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, Del.) 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 3200 g ae/ha).

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.

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.

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.

6.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-1 (v3) (plant optimized gene). T_1 transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 400,000 T_1 seed were screened and 493 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.12%. Depending on the lot of seed tested, this ranged from 0.05-0.23% (see Table 15). A small lot of AAD-1 (v2) (native)-transformed seed were also selected using the glufosinate selection agent. Two hundred seventy eight glufosinate-resistant T_1 individuals were identified out of 84,000 seed screened (0.33% transformation/selection frequency).

TABLE 15

Selection of AAD-1 (v3) (plant optimized), or AAD-1 (v2) (native), AAD-2 (v1) (native), or plant optimized AAD-2 (v2)-transformed T_1 individual plants using glufosinate and 2,4-D.

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

[1]Glufosinate selection scheme: 280 g 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

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 (6× 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.

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

TABLE 16

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

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

TABLE 16-continued

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	Std
Averages	<20%	20-40%	>40%	Ave	Dev
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

Table 17 shows a similar dose response of T_1 *Arabidopsis* to the phenoxypropionic acid, dichlorprop. Similar trends were seen as with 2,4-D, indicating the chiral propionic side chain indeed serves as an acceptable substrate. Next, it was determined that a degree of increased haloxyfop tolerance could be imparted to transformed *Arabidopsis* at elevated rates of 400-1,600 g ae/ha (Table 18). Normal field use rate for haloxyfop (a grass-specific herbicide) is around 50-70 g ae/ha. Dicots are generally considered naturally tolerant to AOPP herbicides; however, severe physiological effects do occur in *Arabidopsis* at these elevated rates. Some AAD1 (v3) transformed individuals did exhibit increased tolerance to haloxyfop. This provides the first in planta data that AAD-1 (v3) will provide AOPP resistance. No resistance was observed with fluroxypyr (a pryridyloxyacetate auxin) in transformed *Arabidopsis*, consistent with in vitro work using heterologously expressed enzyme.

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.

	% Injury			% Injury		
Averages	<20%	20-40%	>40%	Ave	Std Dev	Range
AAD-1 v3						
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						
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						
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

TABLE 17-continued

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

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.

	% Injury			% Injury	Std	
Averages	<20%	20-40%	>40%	Ave	Dev	Range
AAD-1 v3						
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						
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						
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

6.6—AAD-1 (v3) as a Selectable Marker.

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 (50 g 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.

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.

6.7—Heritability.

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 ($P \geq 0.05$).

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.

6.8—Additional Herbicide Resistance Attributable to AAD-1 in *Arabidopsis*.

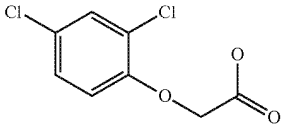
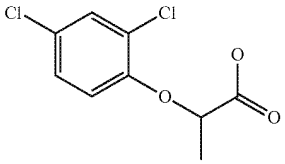
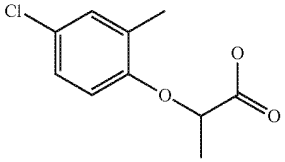
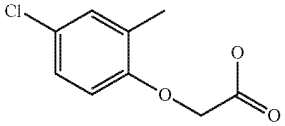
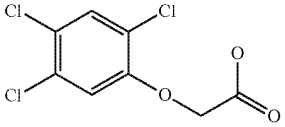
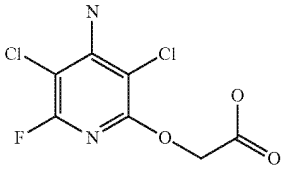
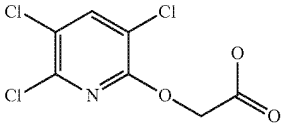
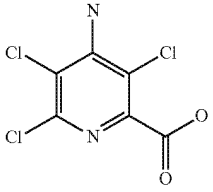
The ability of AAD-1 (v3) to provide resistance to other aryloxyphenoxyalkanoate herbicides in transgenic *Arabidopsis* was determined using a modified in vitro plate assay. Seeds from wild-type *Arabidopsis thaliana* as well as *Arabidopsis thaliana* containing the plant optimized AAD-1 (v3) gene (T_4 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.

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, N.J.). 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).

Assessment of growth reduction. The apical portion of the *Arabidopsis* plants grown in the treated media was assessed visually relative to the apical portion of the plants grown in the media containing only DMSO. Values were recorded as % growth reduction. Assessments of root growth inhibition of the *Arabidopsis* plants grown in the treated media were achieved by carefully extracting the plants from the media and measuring the length of the root. These root lengths were then compared to the root length of the control plants to determine a % growth reduction. A minimum of five plants were assessed for each treatment. The values recorded are an average of all the plants assessed. The calculated concentration to reach 50% inhibition effect (I_{50}) were determined for both root and shoots of wildtype and AAD-1-transformed *Arabidopsis*. The ratios of resistant to sensitive biotypes are included in Table 19. A ratio>2 for both root and shoot measurements generally signifies significant resistance. The higher the ratio, the greater the level of resistance. All commercial phenoxy auxins showed significant levels of resistance including oxyacetic acids (2,4-D and MCPA) as well as oxypropionic acids (dichlorprop and mecoprop). In fact, the chronic root assessment shows resistance to the oxypropionic acid is higher with AAD-1 (v3) than for the oxyacetic acids, consistent with enzymatic characteristics of AAD-1 (v1). Assessment of other auxins containing pyridine rings showed AAD-1 (v3) did not effectively protect *Arabidopsis* form pyridyloxyacetates herbicides, triclopyr and fluroxypyr, or the picolinic acid herbicide, picloram. The broad phenoxy auxin resistance is the first reported in planta. The alternative auxins to which AAD-1 does not protect would be viable tools for the control and containment of AAD-1-transformed commercial crops or experimental plant species.

TABLE 19

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

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

6.9—Foliar Applications Herbicide Resistance in AAD-1 *Arabidopsis*.

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.

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.

	Ave % Injury 14DAT	
Herbicide Treatment	AAD1.01.315.076.T_4 homozygous AAD1 plants	PatCry1f-Control
Phenoxypropionic auxins		
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		
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
Pyridyloxyacetic auxins		
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

6.10—Relationship of Plant Growth to AAD-1 (v3) Expression in *Arabidopsis*.

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. FIGS. **8**A and **8**B 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.

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 (FIG. **9**A). These plants also expressed high level of AAD-1 (v3), from 0.1 to 0.25% of (FIG. **9**B). 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 (FIG. **9**A), 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.

6.11—Molecular Analysis of AAD-1 (v3) *Arabidopsis*.

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.

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× 100 microjoules. The membrane was then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane was baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment consisted of the prepared primers (Pat 5-3 AGATACCCTTGGTTGGTTGC) (SEQ ID NO:23) and (Pat 3-3 CAGATGGATCGTTTGGAAGG) (SEQ ID NO:24) designed to obtain the coding region of PAT. The product was run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM per ml of Perfect Hyb buffer was used to hybridize the southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to film overnight, incubating at −80° C.

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.

6.12—*Arabidopsis* Transformed with Molecular Stack of AAD-1 (v3) and Glyphosate Resistance Gene.

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 FIG. **10**). AAD-1 (v3) did not provide resistance to glyphosate itself (re: pDAB721 response). These T_1 plants will be grown to seed, self-pollinated to yield T_2 seed. The pDAB 3230 T_1 plants have demonstrated tolerance to lethal doses of 2,4-D and glyphosate. T_2 plants will be further tested to demonstrate these co-transformed plants will withstand glyphosate+2,4-D treatments applied in tankmix as described in Example 21 and shown for AAD-1 (v3)-transformed corn in Example 8.

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

7.1—Cloning of AAD-1 (v3).

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.

7.2—Callus/Suspension Initiation.

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.

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.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L α-naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines

typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

7.3—Cryopreservation and Thawing of Suspensions.

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.

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 100×25 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.

7.4—Dose Response of Non-Transformed Tissue to Haloxyfop Acid.

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 60×20 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 FIG. **11**.

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 FIG. **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).

7.5—WHISKERS-Mediated Transformation Using Bialaphos Selection.

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

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.

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 60×20 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.

After 1 week, filter papers were transferred to 60×20 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).

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 100×15 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.

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 60×20 mm

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

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 150×25-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.

7.6—WHISKERS-Mediated Transformation Using Haloxyfop Selection.

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.

After 0-7 days on GN6 selection-free medium, filter papers were transferred to 60×20-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.

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.

7.8—Quantitative Demonstration of In Vitro Tolerance from AAD-1 (v3) Expressing Callus Events from Bialaphos Selection

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 To 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	+	+

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 FIG. **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.

7.9—WHISKERS-Mediated Transformation Using Imazethapyr Selection.

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.

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	+++

TABLE 22-continued

AAD-1 Event ID Number	Expression Level (ppm)	Western Blot
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)		++++

7.10—Molecular Analysis: Maize Materials and Methods.

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.

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.

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.

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 AfI 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 20×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 cross-linker (Stratagene UV stratalinker 1800) at 1200×100 micro-joules. 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

8.1—Tolerance of to 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 (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 1× lethal dose (~⅛× field dose) up to 8× field doses (64× 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.

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 8× field dose (64× 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.

The high level of AOPP tolerance was demonstrated in most events, even when applications were made to plants just coming out of tissue culture (To 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.

FIG. **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.

Table 23 shows data for the responses of selected AAD-1 (v3)-transformed T_0 corn events to three AOPP herbicides applied postemergence.

TABLE 23

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

Construct	Event	Clone	Haloxy-fog* g ae/ha	Cyhalo-fop** g ae/ha	Quizalo-fop*** 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

TABLE 23-continued

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

Construct	Event	Clone	Haloxy-fog* g ae/ha	Cyhalo-fop** g ae/ha	Quizalo-fop*** g ae/ha	% Injury 14 DAT
3404	038	022	(lethal dose = 8.8 g ae/ha)	(lethal dose = 62.5 ae/ha)	70 (lethal dose = 4.4 g ae/ha)	80

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

8.2—Field Tolerance of pDAB3404 T_1 Corn Plants to Quizalofop 2,4-D, and Glufosinate Herbicides.

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 AMD-1 (v3) event lines. The hybrid Croplan 585SR is a sethoxydim resistant line.

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.

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.

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.

8.2.1—Results.

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

TABLE 24

	Treatment (rate) =												
	Quizalofop (140 g ae/ha)		2,4-D amine (1120 g ae/ha)		2,4-D amine (1120 g ae/ha)		Analysis Results Evaluation =			Resistance Segregation Ratios following Liberty Spray		
Event or Hybrid	% 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
	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 ($P > 0.05$)

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.

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.

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).

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 FIG. **16**.

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.

8.3—Stacking of AAD-1 (v3) and PAT with Glyphosate Resistance Genes in Corn.

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.

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.

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 combina-

tions of the three. All applications were formulated in 200 mM Hepes buffer (pH 7.5). In spray applications where glufosinate was present the treatment was formulated with the addition of 2% w/v ammonium sulfate.

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

		% Injury at 14 DAT	
	Field Rate	Hi II X 5XH751 Ave	RR/PAT/ AAD1 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

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.

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 deionized water.

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.

8.3.2—Tolerance of (pDAB3403) To 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 (1× field rate, 4× 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 4× lethal dose of quizalofop at the whole plant level.

8.3.3 Expression of AAD-1 (v3) with Respect to Quizalofop Tolerance.

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 FIG. **15**.). The highest tolerance line, event 3404-074, always expressed with a higher amount of AAD-1 (v3) than the other two events at 1× 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 1× 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)

9.1—Plant Material.

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.

9.2—Tissue Source.

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.

9.3—Preparation of a Superbinary Vector.

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.

9.4—Bacterial Supply.

All transformations use the "Super Binary" vector from Japan Tobacco described in U.S. Pat. No. 5,591,616 ("Method for Transforming Monocotyledons"). To prepare the *Agrobacterium* suspension for treatment, 1-2 loops of pDAS1421 recombinant bacteria from a YP streak plate was put into 5 ml of LS-inf. Mod medium (LS Basal Medium (Linsmaier and Skoog, 1965), N6 vitamins, 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36.0 g/L glucose, 6 mM L-proline, pH 5.2). The mixture was vortexed until a uniform suspension was achieved. The bacterial concentration was taken using a Klett-Summerson Photoelectric Colorimeter by reading the density of the solution. The solution was adjusted to a concentration of Klett 200 (~1×10^9 cfu/ml) and 100 μM actetosyringone added to the solution.

9.5—Infection and Cocultivation.

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.

9.6—Dose Response Using Immature Embryos.

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 FIG. **17**.

9.7—Selection.

After co-cultivation, the embryos were moved through a 2-step selection scheme after which transformed isolates were obtained. For selection, LSD Mod medium (LS Basal Medium, N6 vitamins, 1.5 mg/L 2,4-D, 0.5 g/L MES, 30.0 g/L sucrose, 6 mM L-proline, 1.0 mg/L $AgNO_3$, 250 mg/L cephotaxime, 2.5 g/L Gelrite, pH 5.7) was used along with one of two selection levels of either haloxyfop, cyhalofop, or imazethapyr. Throughout the selection phase, the embryos are cultured in the dark at 28° C. The embryos were first transferred to an initial level of selection (50-100 nM R-haloxyfop or 300 nM R-cyhalofop) for 14 days, then moved up to a higher selection level (250-500 nM R-haloxyfop acid or 1.5 μM cyhalofop) at a rate of 5 embryos/plate. A subset of embryos were similarly stepped-up from 100 to 500 nM imazethapyr from Pursuit® DG as a positive control. Pursuit© is used as the chemical selection agent when the AHAS gene is used, based on U.S. Pat. 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.

9.8.—Regeneration and Seed Production.

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.

9.9—Event Recovery and Analysis Particulars; Whole Plant Screening of to Corn Lineages Containing AAD-1 (v3) and AHAS (pDAS1421).

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

	In vitro Selection agent	Southern Copy # (callus)	Western Blot (AAD-1) T_0	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop	
Corn event	(nM)	AAD-1	Callus	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

TABLE 26-continued

Corn event	In vitro Selection agent (nM)	Southern Copy # (callus) AAD-1	Western Blot (AAD-1) T_0 Callus	Assessment of T_0 lineages resistant to 35 g ae/ha quizalofop Resistant	Sensitive
1421[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

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

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 (24,000 g) 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

Plasmid pDAB3203 containing the AAD-1 (v1) gene was maintained frozen at −80° C. in TOP10F' cells (Invitrogen) as Dow Recombinant strain DR1878. For expression, plasmid DNA purified from TOP10F' cell culture using Promega's Wizard Kit (Fisher cat. #PR-A1460) was transformed into BL-21 Star (DE3) cells (Invitrogen cat. #C6010-03) following manufacturer's protocol. After transformation, 50 μL of the cells were plated onto LB S/S agar plates, and incubated overnight at 37° C. All colonies from the entire agar plate were scraped into 100 mL LB in a 500 mL tribaffled flask and incubated at 37° C. with 200 rpm shaking for 1 hr. Gene expression was then induced with 1 mM IPTG, and incubated for 4 hrs at 30° C. with 200 rpm shaking. All 100 mL of culture was centrifuged at 4000 rpm for 20 min. The supernatant were then discarded, and the pellets were resuspended in 200 mL of extraction containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 mL of Protease Inhibitor Cocktail (Sigma), and disrupted by ultrasonication treatment on ice using a Branson sonifier. The lysate was centrifuged at 24,000×g for 20 min to remove cell debris. The supernatant containing the AAD-1 protein was then subjected to purification protocol.

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.

Five milligrams purified AAD-1 (v1) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) 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).

11.1—Extracting AAD-1 (v3) from Plant Leaves.

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 5,000× g. The supernatant containing the plant soluble proteins were analyzed for both total soluble protein (TSP) and AAD-1 (v3) concentrations.

11.2—Bradford Assay.

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).

11.3—Enzyme Linked Immuno-Sorbent Assay (ELISA).

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).

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

11.4—Western Blotting Analysis.

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

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, Fla.) 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.

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 $\mu E/m^2s^1$).

Prior to propagation, To 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.

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

TABLE 27

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

Event	PAT copy number (Southern)	Coding Region PCR for AAD-1	ELISA (μg AAD-1/ml plant extract)	Relative tolerance T0 spray with 2,4-D *
721(1)1	1	+	0.9	Medium
721(2)1	nd	nd	0.6	Medium
721(2)2	5	+	0.3	Low
721(2)3	3	+	2.6	Medium

TABLE 27-continued

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(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

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.

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.

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.

To 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 1.5× 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 (~6× 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).

Verification of High 2,4-D Tolerance.

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 187 L/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 200 mM 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 1× field rates (560 g ae/ha) for 2,4-D or R-dichlorprop in either event. Very little injury was observed even up to 8 times field rates (4480 g ae/ha) and this was exhibited as stunting, not auxinic herbicide damage. These results indicated commercial level tolerance can be provided by AAD-1 (v3), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to both chiral (2,4-dichlorophenoxypropionic acid) and achiral (2,4-dichlorophenoxyacetic acid) phenoxy auxin herbicides alone or in tank mix combination.

TABLE 28

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

	Average % Injury of Replicates 14 DAT		
Herbicide	KY160-Wildtype	721(2)013.010 (medium tolerance in T_0 generation)	721(3)008.005 (high tolerance in T_0 generation)
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

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.

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

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.

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).

13.1—Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

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).

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.

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.

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 100×25 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).

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.

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.

Differentiating explants are subsequently transferred to shoot elongation medium for an additional 4 to 10 weeks under the same glufosinate selection or under decreased 2,4-D selection pressure ranging from 10 mg to 100 mg/L. The elongation medium consisted of B5 medium (Sigma cat. #M0404) amended with 1 mg/L zeatin riboside, 0.1 mg/L IAA (indole-3-acetic acid), 0.5 mg/L GA3, 50 mg/L glutamine, 50 mg/L asparagine, and 3% (w/v) sucrose, pH 5.8. Elongated shoots should be rooted, without further selection, on half-strength MS/B5 medium with full-strength vitamins plus 0.5 mg/L NAA (α-naphthaleneacetic acid) or 0.1 mg/L IAA and 2% (w/v) sucrose.

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.

13.1.3—Progeny evaluation. To 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, To 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.

13.2—Transformation Method 2: "No-Shake" *Agrobacterium* Mediated Trans-Formation of Non-Regenerable Soybean Suspension Cells.

The DAS Soybean cell suspensions were cultured on a 3 d 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.

Aliquots of 4 ml of the suspension cells at 0.72 OD^{600} was transferred along with 200 μM acetosyringone (AS) onto a 100×25 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.

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 100×15 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 5× concentration of glufosinate ammonium and were maintained for up to 4 months.

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 (FIG. **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%

13.3—Transformation Method 3: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

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

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 ½ B5G medium where they continued development into plantlets; and these plantlets were removed from the tubes and placed into pots.

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 ½ B5G which contained all ingredients of B5G medium at half strength.

TABLE 30

Growth Media for Soybean

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

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

14.1—Cotton Transformation Protocol.

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.

Cotyledon segments (~5 mm) 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.

Agrobacterium preparation. Inoculate 35 ml 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 108 colony forming units per mL with a total volume of 40 ml.

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.

Larger, well-developed embryos are isolated and transferred to DK media (Table 31) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

TABLE 31

Media for Cotton Transformation

Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1 mM)		1 ml	1 ml	1 ml	4.6 ml		0.5 ml	
2,4-D (1 mM)		1 ml	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 (10 mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

14.2—Experiment Specifics.

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.

14.3—Callus Analysis Results.

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
1	2	pos	***
2	1	pos	***
3	1	pos	***
4	2	pos	***
5	1	pos	***
6	1	pos	neg
7	1	pos	****
8	1	pos	*****
9	2	pos	*
10	1	pos	*
11	1	pos	*****
12	1	pos	*

TABLE 32-continued

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	****

TABLE 32-continued

Analysis of cotton callus

Line	Copy #	PTU	Western
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	**
43	1	pos	*****
44	low DNA	pos	*****
45	1	pos	*****
46	2	pos	*****
47	1	pos	***
48	1	pos	***
49	3	faint pos	neg
50	1	pos	****
51	4	pos	neg
52	2	pos	****
53	1	pos	***
54	1	pos	****
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	*
standard	AAD1	5 ug/ml	*****
standard	AAD1	0.5 ug/ml	**

14.4—Plant Regeneration.

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.

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

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.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform AAD-1 (v3), for example, into these and other plants, including but not limited to Maize (Gramineae *Zea mays*), Wheat (Pooideae *Triticum* spp.), Rice (Gramineae *Oryza* spp. and Zizania spp.), Barley (Pooideae *Hordeum* spp.), Cotton (Abroma Dicotyledoneae *Abroma augusta*, and Malvaceae *Gossypium* spp.), Soybean (Soya Leguminosae *Glycine max*), Sugar beet (Chenopodiaceae *Beta vulgaris altissima*), Sugar cane (Arenga *pinnata*), Tomato (Solanaceae *Lycopersicon esculentum* and other spp., Physalis ixocarpa, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato, Sweet potato, Rye (Pooideae *Secale* spp.), Peppers (Solanaceae *Capsicum annuum, sinense*, and *frutescens*), Lettuce (Compositae *Lactuca sativa, perennis*, and *pulchella*), Cabbage, Celery (Umbelliferae *Apium graveolens*), Eggplant (Solanaceae *Solanum melongena*), *Sorghum* (all *Sorghum* species), Alfalfa (Leguminosae *Medicago sativum*), Carrot (Umbelliferae *Daucus carota sativa*), Beans (Leguminosae *Phaseolus* spp. and other genera), Oats (*Avena Sativa* and *Strigosa*), Peas (Leguminosae *Pisum, Vigna*, and *Tetragonolobus* spp.), Sunflower (Compositae *Helianthus annuus*), Squash (Dicotyledoneae *Cucurbita* spp.), Cucumber (Dicotyledoneae genera), Tobacco (Solanaceae *Nicotiana* spp.), *Arabidopsis* (Cruciferae *Arabidopsis thaliana*), Turfgrass (*Lolium, Agrostis*, and other families), and Clover (Leguminosae). Such plants, with AAD-1 (v3) genes, for example, are included in the subject invention.

Example 16—Stacking AAD-1 (v3) with AHAS Herbicide Resistance Gene

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

17.1—AAD-2 (v1) 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 (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.

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at −80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward: 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse: 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 (added a Xho I site)].

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.

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.

17.2—Completion of AAD-2 (v1) Binary Vector.

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.*

17.3—Comparison of Substrate Specificities of AAD-2 (v1) and AAD-1 (v1)

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. FIG. **22** shows that the relative AAD-2 (v1) activity on the substrates was 2,4-D=dichlorprop>(R,S)-haloxyfop>>(R)-haloxyfop. Thus AAD-2 (v1) differs from AAD-1 (v1) in that it has a similar level of activity on 2,4-D as on dichlorprop (whereas the activity of AAD-1 (v1) on 2,4-D is ~10% that of dichlorprop). AAD-2 (v1) also differs from AAD-1 (v1) in that it is unable to act on (R)-haloxyfop. Table 33 shows data from additional substrates tested with AAD-1 (v1) and AAD-2 (v1) that confirm that AAD-2 (v1) is specific for (S)-enantiomer substrates, in contrast to AAD-1 (v1) which is specific for (R)-enantiomers. In another test, AAD-2 (v1) was found to differ from AAD-1 (v1) in that it releases little or no detectable phenol from 2,4-D sulfonate (in which a sulfonate group replaces the carboxylate of 2,4-D) whereas AAD-1 (v1) produces significant levels of phenol from this compound (~25% of 2,4-D.)

TABLE 33

Comparison of AAD1 and AAD2 activity on various substrates.

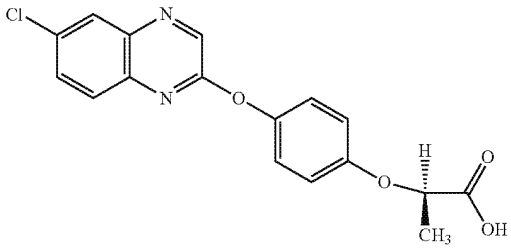
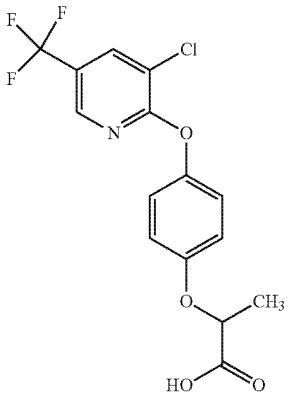
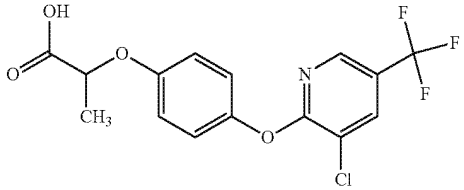
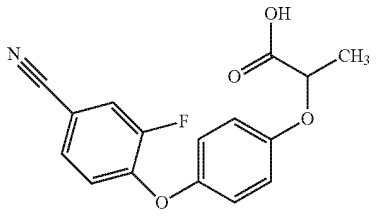
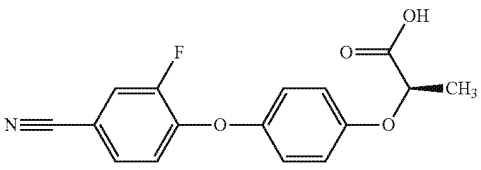
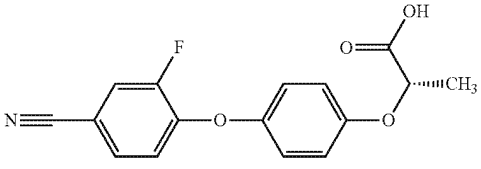
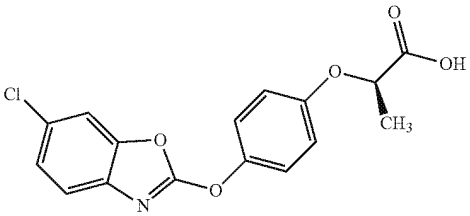
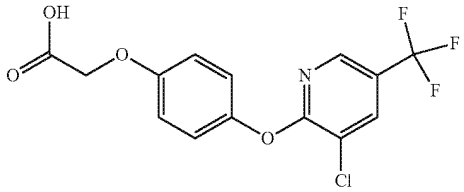
STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
	18706	quizalofop	R	0.27	0.01
	8671	haloxyfop	R	0.12	0
	66905	haloxyfop	R,S	0.1	0.3
	14623	cyhalofop	R,S	0.12	0.1
	14603	cyhalofop	R	0.14	0
	7466	cyhalofop	S	0	0.15

TABLE 33-continued

Comparison of AAD1 and AAD2 activity on various substrates.

STRUCTURE	Reg ID	Compound	Enantiomer	A510 AAD1	A510 AAD2
 Chiral	11044492	fenoxaprop	R	0.14	0
	43865	haloxyfop-acetate	—	0	22

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).

The enzyme kinetics of partially purified AAD-1 (v1) and AAD-2 (v1) were compared using 2,4-D as substrate. See FIG. **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.

17.4—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 (v1) gene was allowed to dry for 7 days at room temperature. T_1 seed was sown in 26.5×51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), 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.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) 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).

Five to six days after planting (DAP) and again 10 DAP, T_1 plants (cotyledon and 2-4-lf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 5-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before. Domes were subsequently removed and plants moved to the greenhouse (22±5° C., 50±30% RH, 14 hours light:10 dark, minimum 500 $\mu E/m^2s^1$ natural+supplemental light) at least 1 day prior to testing for the ability of AAD-1 (v3), AAD-1 (v2), or AAD-2 (v1) to provide phenoxy auxin herbicide resistance.

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, Del.) 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).

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).

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.

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 FIG. **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).

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 FIG. **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

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-1 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2003 or online at cdms.net/manuf/manuf.asp). With AAD-1 (v3) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosinate+2,4-D by utilizing products described in federal herbicide labels (CPR, 2003) and uses described in Agriliance Crop Protection Guide (2003), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-1 (v3) gene if stably transformed.

Example 19—In-Crop Use of Phenoxy Auxins Herbicides in AAD-1 (v3) Only Transformed Soybeans, Cotton, and Other Dicot Crops

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).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds.

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.

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

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-1 (v3) would allow the use of highly efficacious AOPP graminicides in crops normally sensitive to these herbicides. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D, dichlorprop, et al.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing and alternative broadleaf weeds. AAD-1 (v3)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha would be used. A variety of broad spectrum AOPP graminicide herbicides (including but not limited to haloxyfop, quizalofop, fenoxaprop, and fluazifop) could be utilized for effectively controlling a wide selection of grass weeds. Cyclohexanedione graminicidal herbicides like sethoxydim, clethodim, et al. could not be used in this system as shown for dicot crops since AAD-1 would not protect from this chemistry, and grass crops will be naturally sensitive to the cyclohexanedione chemistries. However, this attribute would enable the use of cyclohexanedione herbicides for the subsequent control of AAD-1 (v3)-transformed grass crop volunteers. Similar weed control strategies are now enabled by AAD-1 for the dicot crop species. A combination of quizalofop (20-100 g ae/ha) plus 2,4-D (420-840 g ae/ha) could provide two herbicide modes of action in an AAD-1 (v3) transformed monocot crop (e.g., corn and rice) that would control most agronomic weeds in a similar fashion to glyphosate in glyphosate tolerant crops (see weed control spectra by reference to Agriliance Crop Protection Guide performance ratings).

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D and/or AOPP herbicides when used at higher rates. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-1 (v3) HTC rotation strategy, whether one rotates crops species or not. Additionally, the grass and broadleaf weed control components of this system are independent of one another, thus allowing one skilled in the are of weed control to determine the most cost effective and efficacious ratio of auxin and AOPP herbicide. For example, if broadleaf weeds were the only significant weeds present when herbicide applications were needed, an herbicide application of 560 to 1120 g ae/ha 2,4-D could be made without another herbicide. This would reduce unnecessary herbicide applications, provide flexibility to reduce input costs and reduce environmental loads of pesticides, and reduce unnecessary selection pressure for the development of herbicide resistant weeds. The increased tolerance

of corn, and other monocots to the phenoxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots.

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

The vast majority of cotton, canola, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; isb.vt.edu/cfdocs/fieldtests1.cfm, 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-1 (v3) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a GT trait are stacked in any monocot or dicot crop species:

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

b) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant grass species like *Lolium rigidum* or *Eleusine indica,* 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control.

c) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AMD-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.

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

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).

By stacking AAD-1 (v3) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can

selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.
   i) Inherently tolerant grass weed species (e.g., *Echinochloa* spp or *Sorghum* spp) could be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
   ii) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis* and *Apocynum cannabinum*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species.

b) A three-way combination of glufosinate (200-500 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

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

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like AHAS et al. Imidazolinone tolerant HTCs to date have the advantage of being non-transgenic. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agriliance, 2003). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2004). By stacking AAD-1 (v3) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-1 (v3), one can selectively apply AOPP herbicides in monocot crops, monocot crops will have a higher margin of phenoxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-1 (v3) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.
   i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis, Ambrosia trifida, Chenopodium album* (among others, Heap, 2004) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
   ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D.
   iii) ALS-inhibitor resistant grass weeds like *Sorghum halepense* and *Lolium* spp. can be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.
   iv) Inherently tolerant grass weed species (e.g., *Agropyron repens*) could also be controlled by tank mixing 10-200 g ae/ha (preferably 20-100 g ae/ha) quizalofop.

b) A three-way combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+2,4-D (280-1120 g ae/ha)+quizalofop (10-100 g ae/ha), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

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

24.1—Media Description.

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 100×20 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).

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 (½MSH50).

24.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa* L. *japonica* cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week prior to storage at 4° C.

24.3—Microprojectile Bombardment.

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).

24.4—Tolerance Testing.

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

24.5—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 scanned in the florometer (BioTek) with known standards to obtain the concentration in ng/µl.

24.6—Southern Blot Analysis.

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×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 1200×100 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. 20 mM 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—GGGCAGGCCTAACTC-CACCAA) (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.

The product was run on a 1% agarose gel and excised then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane was then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling reaction (Stratagene 300392) procedure was used to develop the p32 based probe (Perkin Elmer). The probe was cleaned-up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM were used to hybridize the Southern blots overnight. After the overnight hybridization the blots were then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots were then exposed to a phosphor image screen overnight and scanned using a storm scanner (MOLECULAR DEVICES). A summary of the results is presented in Table 36.

TABLE 36

Southern Results.		
Event	Integration Southern data Number of bands	PTU Southern data Expected size 3049bp
63-1 A	8	yes, 7 distinct bands
63-1 F	5	yes, 9 distinct bands
63-4 A	20	yes, 20 distinct bands
63-4 D	20	yes, 19 distinct bands
63-6 C	2	Insufficient DNA yield for both cuts

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.

24.7—Western Data

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

			ELISA		
Lane	Sample Name	TSP (μg/mL)	[AAD1] (ng/mL)	Expression (ppm)	Western
1	Control	6719.58	0.00	0.00	—
2	63-1A	8311.87	351.17	42.25	±
3	63-1F	11453.31	2092.35	182.69	++
4	63-4B	13835.09	216.00	15.61	+
5	36-4D	13656.49	717.05	52.51	++
6	63-6C	5343.63	0.00	0.00	—
7	AAD1 Standard (0.5 μg/mL)				+++
8	AAD1 Standard (5.0 μg/mL)				+++++

Example 25—Turf Grass Transformation Procedures

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).

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.

25.1—Seed Preparation for Embryogenic Callus Induction.

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).

25.2—Embryogenic Callus Induction.

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.

25.3—*Agrobacterium* Infection and Co-Cultivation.

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.

25.4—Resting Stage and *Agrobacterium* Control.

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.

25.5—Selection and Potential Transgenic Colony Identification.

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.

25.6—Regeneration of Transgenic Plants.

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).

25.7—Root Induction and Transfer to Greenhouse.

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.

25.8—Vernalization and Out-Crossing of Transgenic Plants.

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.

25.9—Other Target Grasses.

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

26.1—Canola Transformation.

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.

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.

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.

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

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).

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.

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.

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.

26.2—"Molecular Analysis": Canola Materials and Methods

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

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

26.2.2—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 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.

26.3—ELISA.

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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gagccgctcg acgacagcac ctggaacgaa atcctcgacg cgttccacac ttaccaggtc 180

atctattttc ccggccaggc gatcaccaac gaacagcaca tcgccttcag ccggcgcttc 240

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caggccacca tcgaagggtt gaacgtagtg cacagcgcca cgcgtgtgtt cggctcgctc 540

taccaggccc agaaccggcg cttcagcaac accagcgtca aggtgatgga cgtcgacgcg 600

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<223> OTHER INFORMATION: primer linker
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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

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<223> OTHER INFORMATION: primer linker
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<220> FEATURE:
<221> NAME/KEY: misc_feature
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<223> OTHER INFORMATION: primer linker

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acagcacctg gaatgagata ttggatgcct tccacactta ccaagtcatc tactttcctg 180

gccaagcaat caccaatgag cagcacattg cattctcaag aaggtttgga ccagttgatc 240

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ccaatgagtc tggaagggtg attggtgatg actggcacac agactccact ttccttgatg 360

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aagggctcaa cgttgtgcac tctgccacac gtgtgttcgg ttccctctac caagcacaga 540

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<223> OTHER INFORMATION: 3'saci primer

<400> SEQUENCE: 7

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<400> SEQUENCE: 8

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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

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    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> SEQ ID NO 10
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<212> TYPE: PRT
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<223> OTHER INFORMATION: AAD-1 v2 Translation
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<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Different from v1
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<223> OTHER INFORMATION: Different from v1

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

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

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

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

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

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

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

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

Gly Gly Val Arg Pro Ala Arg
    290                 295


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<223> OTHER INFORMATION: Different from V1

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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
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        195                 200                 205

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

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

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

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

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

Val Gly Gly Val Arg Pro Ala Arg
    290                 295


<210> SEQ ID NO 12
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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


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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
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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
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Val

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<220> FEATURE:
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<213> ORGANISM: Glycine max

<400> SEQUENCE: 20

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

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

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

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

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

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

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

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

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

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

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

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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> SEQ ID NO 21
<211> LENGTH: 525
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Doubly mutated Soybean EPSPS protein: threonine
      183 converted to isoleucine; proline 187 converted to serine

<400> SEQUENCE: 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
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           20                  25                  30

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

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

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

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

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

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

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

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

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

Phe Leu Gly Asn Ala Gly 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
        435                 440                 445
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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
            500                 505                 510

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


<210> SEQ ID NO 22
<211> LENGTH: 1604
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Soybean-biased DNA sequence encoding
      doubly-mutated EPSPS disclosed in SEQ ID NO:21, with added
      sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1575)
<223> OTHER INFORMATION: Coding sequence of soybean-biased DNA sequence
      encoding the EPSPS protien of SEQ ID NO:21
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1576)..(1578)
<223> OTHER INFORMATION: Translation terminator tga
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1579)..(1604)
<223> OTHER INFORMATION: Sequence included to introduce translation stop
      codons in all six open reading frames, and to introduce a SacI
      restriction enzyme recognition site for cloning purposes

<400> SEQUENCE: 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
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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 aacccccacct gagaagctca atgtgacagc cattgacacc 1440

tatgatgacc acagaatggc aatggctttc tcccttgctg cctgtggtga tgtgcctgtg 1500

actatcaaag accctgggtg cacaaggaag acatttccag actactttga agttttggag 1560

aggttgacaa agcactgagt agttagctta atcacctaga gctc 1604

<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 5-3

<400> SEQUENCE: 23

agataccctt ggttggttgc 20

<210> SEQ ID NO 24
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer Pat 3-3

<400> SEQUENCE: 24

cagatggatc gtttggaagg 20

<210> SEQ ID NO 25
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU forward primer

<400> SEQUENCE: 25

ataatgccag cctgttaaac gcc 23

<210> SEQ ID NO 26
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 PTU reverse primer

<400> SEQUENCE: 26

ctcaagcata tgaatgacct cga 23

<210> SEQ ID NO 27
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward primer for Coding Region PCR AAD-1 (RdpAcodF)

<400> SEQUENCE: 27

atggctcatg ctgccctcag cc 22

-continued

<210> SEQ ID NO 28
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse primer for Coding Region PCR AAD-1 (RdpAcodR)

<400> SEQUENCE: 28

cgggcaggcc taactccacc aa 22

<210> SEQ ID NO 29
<211> LENGTH: 932
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-2 v2 plant-optimized nucleotide

<400> SEQUENCE: 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

<210> SEQ ID NO 30
<211> LENGTH: 296
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Translated AAD-2 v2 protein

<400> SEQUENCE: 30

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

Val Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg
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

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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

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
            260                 265                 270

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

Thr Val Gln Gln Gln Ala Ala Glu
    290                 295


<210> SEQ ID NO 31
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 forward primer

<400> SEQUENCE: 31

atggctcatg ctgccctcag cc                                              22


<210> SEQ ID NO 32
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Southern fragment PCR AAD-1 reverse primer

<400> SEQUENCE: 32

gggcaggcct aactccacca a                                               21


<210> SEQ ID NO 33
<211> LENGTH: 84
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: alpha-ketoglutarate-dependent dioxygenase motif
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
```

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<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (3)..(3)
<223> OTHER INFORMATION: Xaa at position 3 is aspartic acid or glutamic acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(7)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa at position 8 is threonine or serine
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (9)..(78)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (80)..(83)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 33

His Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
50 55 60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa His Xaa
65 70 75 80

Xaa Xaa Xaa Arg

<210> SEQ ID NO 34
<211> LENGTH: 171
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 motif
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(27)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(159)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (161)..(170)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 34

His Xaa Asp Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1 5 10 15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Thr Xaa Xaa Xaa Xaa
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
35 40 45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
50 55 60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa

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65                  70                  75                  80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                85                  90                  95

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
            100                 105                 110

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        115                 120                 125

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
    130                 135                 140

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa His
145                 150                 155                 160

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Arg
                165                 170


<210> SEQ ID NO 35
<211> LENGTH: 175
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAD-1 motif extended
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (4)..(27)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (29)..(159)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (161)..(170)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (172)..(174)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid

<400> SEQUENCE: 35

His Xaa Asp Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
1               5                   10                  15

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Thr Xaa Xaa Xaa Xaa
            20                  25                  30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        35                  40                  45

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
    50                  55                  60

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
65                  70                  75                  80

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                85                  90                  95

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
            100                 105                 110

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        115                 120                 125

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
    130                 135                 140

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa His
145                 150                 155                 160
```

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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Arg Xaa Xaa Xaa Arg
165 170 175

The invention claimed is:

1. A transgenic soybean plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, which are aryloxyalkanoate herbicides, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R$ (SEQ ID NO: 34), wherein

X112 represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114-137}$ represents a sequence of 24 amino acids;

$(X)_{139-269}$ represents a sequence of 131 amino acids; and

$(X)_{271-280}$ represents a sequence of 10 amino acids,

wherein said AAD-1 protein when expressed in a soybean plant cell renders said soybean plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, as compared to an untransformed soybean plant cell.

2. The plant cell of claim 1 wherein said AAD-1 motif has the general formula of: $HX_{112}D(X)_{114-137}T(X)_{139-269}H(X)_{271-280}R(X)_{282-284}R$ (SEQ ID NO: 35), wherein $(X)_{282-284}$ represents a sequence of 3 amino acids.

3. A transgenic soybean plant comprising a plurality of the soybean plant cell of claim 1, wherein expression of said polynucleotide renders said plant tolerant to said aryloxyalkanoate herbicide.

4. The plant of claim 3 wherein said aryloxyalkanoate herbicide selected from the group consisting of 2,4-dichlorophenoxyacetic acid, MCPA, dichlorprop, and mecoprop.

5. The plant of claim 3 wherein said aryloxyalkanoate herbicide is an (R)-aryloxyphenoxypropionate.

6. The plant of claim 3 wherein said aryloxyalkanoate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

7. The plant of claim 3 wherein said plant further comprises a second herbicide resistance gene.

8. The plant of claim 7 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, acetolactate synthase (ALS) inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba, and inhibitors of protoporphyrinogen oxidase (PPO).

9. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 3 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 (R)-aryloxyphenoxypropionate herbicide.

10. The method of claim 9 wherein said phenoxy auxin herbicide is an R-enantiomer of a chiral phenoxy auxin.

11. The method of claim 9 wherein said phenoxy auxin herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

12. The method of claim 9 wherein said (R)-aryloxyphenoxypropionate herbicide is selected from the group consisting of (R)-fluazifop, (R)-haloxyfop, (R)-diclofop, (R)-quizalofop, (R)-fenoxaprop, (R)-metamifop, (R)-cyhalofop, and (R)-clodinofop.

13. The method of claim 9 wherein said method comprises applying a second herbicide.

14. The method of claim 13 wherein said first herbicide and said second herbicide are applied sequentially.

15. The method of claim 13 wherein said first herbicide and said second herbicide are applied concurrently.

16. The method of claim 13 wherein said first herbicide is a phenoxy auxin and said second herbicide is an (R)-aryloxyphenoxypropionate.

17. The method of claim 13 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase (ALS) inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitors.

18. The method of claim 13, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate or glufosinate.

19. The method of claim 13, wherein said first herbicide is an (R)-aryloxyphenoxypropionate and said second herbicide is glyphosate or glufosinate.

20. The method of claim 13 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

21. The method of claim 20 wherein said second herbicide resistance gene is selected from the group consisting of a modified acetolactate synthase (ALS) gene, a glyphosate resistance gene, a glufosinate resistance gene, and a dicamba-degrading enzyme gene.

22. The method of claim 13 wherein said method further comprises applying a third herbicide.

23. The method of claim 22, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

24. The method of claim 23 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

25. A seed comprising a plurality of the plant cell of claim 1.

26. A method of controlling weeds in a field, wherein said method comprises applying an aryloxyalkanoate herbicide to said field and planting a seed of claim 25 in said field within 14 days after applying said aryloxyalkanoate herbicide.

27. A plant grown from the seed of claim 25, wherein said plant comprises said polynucleotide.

28. A part, progeny, or asexual propagate of the plant of claim 27, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

29. An (R)-aryloxyphenoxypropionate herbicide tolerant transgenic soybean plant cell comprising a recombinant polynucleotide that encodes an AAD-1 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity

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enzymatically degrades a phenoxy auxin herbicide and an (R)-aryloxyphenoxypropionate herbicide, further wherein said AAD-1 protein comprises:

i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 9; and

ii) an AAD-1 motif having the general formula of:

$$HX_{112}D(X)_{114\text{-}137}T(X)_{139\text{-}269}H(X)_{271\text{-}280}R$$ (SEQ ID NO: 34), wherein

X112 represents a single amino acid at position 112, relative to the sequence of SEQ ID NO: 9;

$(X)_{114\text{-}137}$ represents a sequence of 24 amino acids;

$(X)_{139\text{-}269}$ represents a sequence of 131 amino acids; and

$(X)_{271\text{-}280}$ represents a sequence of 10 amino acids, wherein said motif has 90% sequence identity with corresponding amino acids of position 111 to 281 of SEQ ID NO: 9

wherein said AAD-1 protein when expressed in a soybean plant cell renders said soybean plant cell tolerant to a phenoxy auxin herbicide and an (R)-aryloxyphenoxy-propionate herbicide, as compared to an untransformed soybean plant cell.

* * * * *

EXHIBIT I

Application for authorisation to place on the market MON 94313 soybean in the European Union, according to Regulation (EC) No 1829/2003 on genetically modified food and feed

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Part VII

Summary of Application

Data protection.
This application contains scientific data and other information which are protected in accordance with Art. 31 of Regulation (EC) No 1829/2003.

1. GENERAL INFORMATION

1.1. Details of application

(a) Member State of application

The Netherlands

(b) Application number

GMFF-2022-6595

(c) Name of the product (commercial and any other names)

The development code for this genetically modified (GM) soybean is MON 94313.

MON 94313 single product is not and will not be commercialised on its own, instead stacked products, made via traditional breeding, are the aimed commercial products. Therefore, no commercial name will be attributed to this product and the hypothetical import of this product in the European Union (EU) is considered in this document.

(d) Date of acknowledgement of valid application

Not available at the time of initial submission.

1.2. Applicant

(a) Name of applicant

Bayer Agriculture BV on behalf of Bayer CropScience LP

(b) Address of applicant

Bayer Agriculture BV
Haven 627
Scheldelaan 460
B-2040 Antwerp
BELGIUM

Bayer CropScience LP
700 Chesterfield Pkwy West
Chesterfield, Missouri 63017
U.S.A.

(c) Name and address of the representative of the applicant established in the Union (if the applicant is not established in the Union)

See above.

1.3. Scope of the application

(a) Genetically modified food

☑ Food containing or consisting of genetically modified plants

☑ Food produced from genetically modified plants or containing ingredients produced from genetically modified plants

(b) Genetically modified feed

☑ Feed containing or consisting of genetically modified plants

☑ Feed produced from genetically modified plants

(c) Genetically modified plants for food or feed uses

☑ Products other than food and feed containing of consisting of genetically modified plants with the exception of cultivation

☐ Seeds and plant propagating material for cultivation in the Union

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The scope of this application covers the hypothetical import, processing and all uses of MON 94313 as any other soybean but excludes cultivation.

1.4. Is the product or the uses of the associated plant protection product(s) already authorised or subject to another authorisation procedure within the Union?

No ☑

Yes ☐ (in that case, specify)

1.5. Has the genetically modified plant been notified under Part B of Directive 2001/18/EC?

Yes ☐

No ☑ (in that case, provide risk analysis data on the basis of the elements of Part B of Directive 2001/18/EC)

The risk assessment presented in this application includes data collected from field trials conducted at multiple United States (US) locations covering a range of environmental conditions. A summary of the conclusions of the risk analysis that demonstrate the safety of MON 94313 to humans, animals and the environment, has been presented in the respective sections throughout this summary.

1.6. Has the genetically modified plant or derived products been previously notified for marketing in the Union under Part C of Directive 2001/18/EC?

No ☑

Yes ☐ (in that case, specify)

1.7. Has the product been subject to an application and/or authorised in a third country either previously or simultaneously to this application?

No ☐

Yes ☑ in that case, specify the third country, the date of application and, where available, a copy of the risk assessment conclusions, the date of the authorisation and the scope of the application

MON 94313 regulatory submissions and reviews are currently in progress in selected countries around the world.

1.8. General description of the product

(a) Name of the recipient or parental plant and the intended function of the genetic modification

MON 94313 soybean was developed through *Agrobacterium tumefaciens* mediated transformation of soybean tissues to express MON 94313 DMO, PAT, FT_T.1 and TDO proteins conferring tolerance to dicamba-, glufosinate-, 2,4-D- and mesotrione- based herbicides, respectively.

(b) Types of products planned to be placed on the market according to the authorisation applied for and any specific form in which the product must not be placed on the market (such as seeds, cut-flowers, vegetative parts) as a proposed condition of the authorisation applied for

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(c) Intended use of the product and types of users

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

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(d) Any specific instructions and recommendations for use, storage and handling, including mandatory restrictions proposed as a condition of the authorisation applied for

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(e) If applicable, geographical areas within the Union to which the product is intended to be confined under the terms of the authorisation applied for

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(f) Any type of environment to which the product is unsuited

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(g) Any proposed packaging requirements

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(h) Any proposed labelling requirements in addition to those required by other applicable EU legislation than Regulation (EC) No 1829/2003 and when necessary a proposal for specific labelling in accordance with Article 13(2) and (3), Article 25(2)(c) and (d) and Article 25(3) of Regulation (EC) No 1829/2003. In the case of products other than food and feed containing or consisting of genetically modified plants, a proposal for labelling has which complies with the requirements of point A(8) of Annex IV to Directive 2001/18/EC must be included.

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(i) Estimated potential demand

(i) In the EU

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(ii) In EU export markets

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

(j) Unique identifier in accordance with Regulation (EC) No 65/2004

The OECD unique identifier for MON 94313 is MON-94313-8.

1.9. Measures suggested by the applicant to take in the case of unintended release or misuse of the product as well as measures for disposal and treatment

Not applicable. MON 94313 single product is not and will not be commercialised on its own.

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2. INFORMATION RELATING TO THE RECIPIENT OR (WHERE APPROPRIATE) PARENTAL PLANTS

2.1. Soybean name

(a)	**Family name**	Leguminosae
(b)	**Genus**	*Glycine*
(c)	**Species**	*max.*
(d)	**Subspecies**	Not applicable
(e)	**Cultivar, breeding line**	A3555
(f)	**Common name**	Soybean

2.2. Geographical distribution and cultivation of the plant, including the distribution within the Union

Today, soybean is the most prevalently grown oilseed in over 35 countries worldwide. The largest soybean producer in the European Union (EU) is Italy, followed by Serbia, France, Romania and Austria.

2.3. Information concerning reproduction (for environmental safety aspects)

(a) Mode(s) of reproduction

Soybean is a diploidised tetraploid (2n = 40 chromosomes) and is a self-pollinated species, propagated by seed.

(b) Specific factors affecting reproduction

Soybean is a quantitative short day plant and hence flowers more quickly under short days. As a result, photoperiodism and temperature response are important in determining areas of cultivar adaptation.

During the reproductive stages of development, soybean plants are particularly sensitive to hydric and thermal (low temperature) stress which can cause significant flower abortion and yield loss. Soybean does not yield well on acidic soils and the addition of limestone may be required.

(c) Generation time

The life cycle of soybean is approximately 100 to 160 days, depending on the variety and the region in which it is cultivated.

2.4. Sexual compatibility with other cultivated or wild plant species (for environmental safety aspects)

Potential for cross-pollination with cultivated soybean varieties

Although soybean is a self-pollinated species, natural cross-pollination can occur, at very low rate. Cross-pollination frequencies may vary due to growing season and genotype, and most outcrossing occurs with immediately surrounding plants. Insect activity increases the outcrossing rate, but soybean generally is not the preferred plant for pollinators.

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Potential for cross-pollination with wild species

From a taxonomic standpoint, both the wild annual species of subgenus *Soja* and the wild perennial species of subgenus *Glycine* are candidates for gene exchange with the cultivated soybean. No other genus is related closely enough to soybean to allow for the possibility of outcrossing. There are no known reports of successful natural hybridisation between cultivated soybean and wild perennial species of subgenus *Glycine.* Moreover, there are no wild relatives of subgenus *Glycine* in Europe. The wild annual species *G. soja,* can hybridise naturally with the cultivated soybean, *G. max,* since they are both members of the subgenus *Soja.* Therefore, gene transfer between cultivated soybean and wild species of subgenus *Soja* may occur, but not in Europe, where the wild relatives of subgenus *Soja* are not present.

2.5. Survivability (for environmental safety aspects)

(a) Ability to form structures for survival or dormancy

Cultivated soybean plants are annuals, and they reproduce solely by means of seeds. Mature soybean seeds do not have pronounced seed dormancy, are sensitive to cold and are not likely to survive from one growing season to the next if left in the field over winter. Commercial soybean seeds are selected for lack of dormancy, enabling them to germinate quickly under adequate temperature and moisture which could potentially allow them to grow as volunteers in a field. However, volunteers likely would be killed by frost during autumn or winter of the year they were produced. If they did become established, volunteer plants would not compete well with the succeeding crop, and could be controlled readily by either mechanical or chemical means.

(b) Specific factors affecting survivability

See Section 2.5.(a).

2.6. Dissemination (for environmental safety aspects)

(a) Ways and extent of dissemination

In theory, soybean dissemination may occur by means of seed dispersal or pollen dispersal. In contrast to weed species that have various seed dispersal mechanisms, soybean pods and seed do not have dispersal mechanisms that facilitate seed or pod movement over long distances. Furthermore, neither the soybean seedpod, nor the seed have morphological characteristics that would facilitate animal transportation. Primary movement of soybean seed is facilitated by human activities during planting, harvesting and transport of seed; however, few seeds are typically lost due to the relatively large seed size and practices taken to reduce seed loss.

Soybean pollen may also be considered as a means for dissemination, but the pollen viability outside of the soybean flower is limited by the fact that soybean is predominantly a self-pollinated species. The major barrier that prevents dissemination of soybean pollen, and therefore cross-pollination, is the enclosure of both the stigma and anthers within the flower, even during maturation of the pollen. As a consequence, the potential for the pollen to become disseminated is reduced and the chance for self-pollination greatly increases. However, natural cross-pollination may occur to a certain extent as discussed in Section 2.4.

(b) Specific factors affecting dissemination

See Section 2.6.(a).

2.7. Geographical distribution within the Union of the sexually compatible species (for environmental safety aspects)

There are no sexually compatible species of cultivated soybean present in Europe.

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2.8. In the case of plant species not normally grown in the Union description of the natural habitat of the plant, including information on natural predators, parasites, competitors and symbionts (for environmental safety aspects)

Not applicable, as soybean is grown in Europe.

2.9. Other potential interactions, relevant to the genetically modified plant, of the plant with organisms in the ecosystem where it is usually grown, or used elsewhere, including information on toxic effects on humans, animals and other organisms (for environmental safety aspects)

Soybean is known to interact with other organisms in the agricultural environment. Like most crop varieties, it is sensitive to a number of economically important diseases and insect predators and is also susceptible to competition from surrounding weeds. In addition, soybean is involved in the fixation of atmospheric nitrogen into organic nitrogen through a symbiotic association with the bacterium *Bradyrhizobium japonicum*.

Soybean seed is known to contain a number of natural anti-nutritional components, which are completely or partially inactivated during processing. Trypsin (proteinase) inhibitors are known to have anti-nutritive properties in animals fed unprocessed soybean. Other anti-nutrients include lectins, phytic acid, stachyose and raffinose. Some of these anti-nutrients relate to their impact on human nutrition, while others relate to animal nutrition in general, including livestock.

Soybean is one of the eight food groups that are known to elicit food allergic responses in humans. It contains several endogenous proteins that have been shown to elicit an allergenic response when ingested. Relatively few of the specific soybean proteins involved in allergenic reactions in soybean have been uniquely identified or characterised. Allergic responses to soybean are experienced by a very small percentage of the human population, but are considered clinically important. Allergy to soybean is more prevalent in children than adults and is considered a transient allergy of infancy/childhood.

3. MOLECULAR CHARACTERISATION

3.1. Information relating to the genetic modification

(a) Description of the methods used for the genetic modification

MON 94313 was produced by *Agrobacterium*-mediated transformation of soybean tissue.

(b) Nature and source of the vector used

Plasmid Vector PV-GMHT529103 used for the transformation contains two T-DNAs (transfer DNA) that are delineated by Right and Left Border regions and the backbone sequences used during DNA cloning.

(c) Source of donor nucleic acid(s) used for transformation, size and intended function of each constituent fragment of the region intended for insertion

The T-DNA I in PV-GMHT529103, intended for insertion, contains the *dmo, pat, ft_t.1* and *TDO* expression cassettes.

The *dmo* expression cassette contains the following genetic elements: the *ubq3* promoter gene from from *Arabidopsis thaliana*, the targeting sequence of the *APG6* gene from *Arabidopsis thaliana* encoding a HSP101 (heat shock protein) homologue and acts as a transit peptide that directs transport of the protein to the chloroplast, the *dmo* gene for the dicamba mono-oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba resistance

and the *sali3-2-Mt1* 3′ UTR sequence from *Medicago truncatula* (barrel medic) of an aluminum-induced Sali3-2 protein that directs polyadenylation of the mRNA.

The *pat* expression cassette contains the following genetic elements: the *GSP579* promoter and 5′ UTR that has been developed from multiple promoter and 5′ UTR sequences from *Arabidopsis thaliana* that directs transcription in plant cells, the *GSI102* intron that has been developed from multiple intron sequences from *Arabidopsis thaliana* that is involved in regulating gene expression, the *pat* gene for the phosphinothricin N–acetyltransferase (PAT) protein of *Streptomyces viridochromogenes* that confers tolerance to glufosinate and the *Hsp20-Mt1* 3' UTR sequence from *Medicago truncatula* (barrel medic) of a putative *Hsp20* gene encoding a heat shock protein that directs polyadenylation of the mRNA.

The *ft_t.1* expression cassette contains the following genetic elements: the *ubq10-At1* promoter, leader and intron from Arabidopsis thaliana of the polyubiquitin gene *ubq10*, which directs transcription in plant cells, the *ft_t.1* gene which corresponds to a modified version of R-2,4 dichlorophenoxypropionate dioxygenase (*RdpA*) gene of *Sphingobium herbicidovorans* that expresses a modified FOPs and 2,4-D dioxygenase protein (FT_T.1) that confers tolerance to 2,4-D herbicide in soybean, and flanking exon sequence of the *hsp70* gene from *Zea mays* (soybean) and the *guf-Mt2* 3' UTR sequence from an expressed gene of *Medicago truncatula* of unknown function that directs polyadenylation of mRNA.

The *TDO* expression cassette contains the following genetic elements: the *GSP576* promoter and 5′ UTR that has been developed from multiple promoter and 5′ UTR sequences from *Arabidopsis thaliana* that directs transcription in plant cells, the GSI17 intron that has been developed from multiple intron sequences from *Arabidopsis thaliana* that is involved in regulating gene expression, the *TDO* gene for the triketone dioxygenase (TDO) protein of *Oryza sativa* that confers tolerance to mesotrione and the *GST7* 3' UTR that has been developed from multiple 3′ UTR sequences from *Zea mays* (maize) that directs polyadenylation of the mRNA.

3.2. Information relating to the genetically modified plant

3.2.1. *Description of the trait(s) and characteristics which have been introduced or modified*

MON 94313 soybean expresses MON 94313 DMO, PAT, FT_T.1 and TDO proteins, conferring tolerance to dicamba-, glufosinate-, 2,4-D- and mesotrione- based herbicides, respectively.

3.2.2. *Information on the nucleic acid(s) sequences actually inserted or deleted*

(a) The copy number of all detectable inserts, both complete and partial

The molecular characterisation of the genetic modification in MON 94313 demonstrates that a single copy of T-DNA I was stably integrated at a single locus of the soybean genome and that no T-DNA-II, plasmid backbone, or PV-GMHT529103 sequences are present in MON 94313.

(b) In case of deletion(s), size and function of the deleted region(s)

A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5' and 3' flanking sequences of MON 94313 indicates that 40 bases of soybean genomic DNA were deleted during integration of the T-DNA.

(c) Sub-cellular location(s) of insert(s) (nucleus, chloroplasts, mitochondria, or maintained in a non-integrated form) and methods for its/their determination

The Chi square (χ^2) analysis of the segregation pattern, according to Mendelian genetics, is consistent with a single site of insertion into soybean nuclear DNA.

(d) The organisation of the inserted genetic material at the insertion site

All genetic elements (except for the right and left border regions) in the insert are intact and the remaining sequence of the insert is identical to the corresponding sequence in PV-GMHT529103.

(e) In case of modifications other than insertion or deletion, describe function of the modified genetic material before and after the modification, as well as direct changes in expression of genes as a result of the modification

Not applicable.

3.2.3. *Information on the expression of the insert*

(a) Information on developmental expression of the insert during the life cycle of the plant

The MON 94313 DMO, PAT, FT_T.1 and TDO protein expression levels were determined by validated multiplexed immunoassay or enzyme linked immunosorbent assay (ELISA) in forage, grain and flower tissues collected from treated and not treated MON 94313 produced during the 2020 US growing season.

(b) Parts of the plant where the insert is expressed

In terms of food and feed safety, assessment of MON 94313 forage and grain are the most relevant tissues. The expression levels were also measured in flowers, used as surrogate tissue for pollen, required for the human dietary exposure assessment for soybean pollen supplements.

3.2.4. *Genetic stability of the insert and phenotypic stability of the genetically modified plant*

Generational stability analysis demonstrated that the T-DNA insert in MON 94313 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 94313. Segregation analysis corroborates the insert stability and independently establishes the nature of the T-DNA as a single chromosomal locus that shows an expected pattern of inheritance.

3.2.5. *Information (for environmental safety aspects) on how the genetically modified plant differs from the recipient plant in:*

(a) Mode(s) and/or rate of reproduction

MON 94313 shows no difference to the conventional counterpart in its phenotypic and agronomic behaviour, except for the herbicide tolerance traits.

(b) Dissemination

See Section 3.2.5.(a).

(c) Survivability

See Section 3.2.5.(a).

(d) Other differences

See Section 3.2.5.(a).

3.2.6. *Any change to the ability of the genetically modified plant to transfer genetic material to other organisms (for environmental safety aspects)*

(a) Plant to bacteria gene transfer

None of the genetic elements in MON 94313 have a genetic transfer function. Therefore, no changes are expected in the ability of this soybean to transfer genetic material to bacteria.

(b) Plant to plant gene transfer

Not applicable, the scope of the current application does not include the cultivation of MON 94313 in the EU.

4. COMPARATIVE ANALYSIS

4.1. Choice of the conventional counterpart and additional comparators

MON 94313 was compared to a conventional counterpart with similar genetic background, as well as with other commercially available soybean varieties.

4.2. Experimental design and statistical analysis of data from field trials for comparative analysis

Field trials for comparative analysis were conducted in multiple field sites in major growing areas of the US during 2020 field season. Additionally, commercial reference varieties were included at each field sites to provide reference substances representative for their respective growing regions. Difference and equivalence tests were conducted using statistical models provided in EFSA guidance[1] and according to the 2010 EFSA Scientific Opinion on Statistical considerations for the safety evaluation of GMOs[2].

4.3. Selection of material and compounds for analysis

The key nutrients and other nutritionally important components that were selected for analysis in the compositional studies were chosen on the basis of internationally accepted guidance provided by the OECD[3] on compositional considerations for new varieties of soybean.

Certain characteristics together with environmental interactions were studied to assess for a potential indicator of phenotypic changes.

4.4. Comparative analysis of composition

The compositional assessment of MON 94313 compared to conventional soybean demonstrated that MON 94313 is compositionally similar to conventional soybean and that MON 94313 is not a significant contributor to compositional variability in soybean.

4.5. Comparative analysis of agronomic and phenotypic characteristics

The assessment of the phenotypic, agronomic and environmental interactions of MON 94313 compared to conventional soybean demonstrated that there are no unexpected changes in the phenotype or ecological interactions indicative of increased plant weed or pest potential.

4.6. Effect of processing

MON 94313 has been shown not to be different from conventional soybean, except for its herbicide tolerance traits. The processing of MON 94313 is therefore not expected to be any different from that of conventional soybean.

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1 EFSA, 2011. Guidance for risk assessment of food and feed from genetically modified plants. EFSA Panel on Genetically Modified Organisms (GMO). EFSA journal, 9(5):2150, 1-37. – Accessed on 06 September 2022

2 EFSA, 2010. Scientific Opinion on Statistical considerations for the safety evaluation of GMOs. . The EFSA Journal (2010), 1250, 1-59. – Accessed on 06 September 2022

3 OECD, 2000. Consensus document on the biology of glycine max (L.) merr. (soybean). OECD, ENV/JM/MONO(2000)9, 10. - Accessed on 06 September 2022.

5. TOXICOLOGY

(a) Toxicological testing of newly expressed proteins

The *dmo, pat, ft_t.1* and *TDO* genes are the only genes expressing novel proteins in MON 94313. Therefore, the safety assessment of the newly expressed proteins is focused on the DMO, PAT, FT_T.1 and TDO proteins expressed in MON 94313.

Following the guidelines adopted by the Codex Alimentarius[4], an assessment of the potential toxicity of the newly expressed proteins was conducted and demonstrated that it is unlikely that the MON 94313 DMO, PAT, FT_T.1 and TDO proteins will cause toxicity concerns due to the following considerations:

- The proteins have a demonstrated history of safe use and pose negligible risk to human and aimal health. The DMO protein is derived from *Stenotrophomonas maltophilia*, has a demonstrated long history of safe use and is expressed in other crop products that have been assessed by several global regulatory agencies around the world[5] including the EFSA[6]. The PAT protein is derived from the bacterium *Streptomyces viridochromogenes* wich is not considered pathogenic to plants, humans or other animals and has also been extensively reviewed by several global regulatory agencies around the world[5] including the EFSA[6]. The FT_T.1 protein is derived from the bacterium *Sphingobium herbicidovorans* which is a common gram-negative, rod-shaped, non-motile, non-spore-forming soil bacterium, strictly aerobic and chemo-organotrophic, and not known to be associated with human disease. The TDO protein comes from *Oryza sativa*, a crop plant with long history of exposure in food or feed.
- The proteins do not have biologically relevant structural similarities to known toxins or other biologically active proteins that could cause adverse effects in humans or animals;
- The proteins behave with a predictable tendency toward protein denaturation and loss of functional activity at elevated temperatures, indicating that they are likely to be denatured during soybean processing.
- The proteins are rapidly digested in mammalian gastrointestinal systems. *In vitro* experiments conducted with MON 94313 DMO, PAT, FT_T.1 and TDO proteins demonstrated that all the proteins are rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) under physiological conditions.

Furthermore, the low concentrations of the MON 94313 DMO, PAT, FT_T.1 and TDO proteins in tissues that are consumed provide additional assurance for their safety.

(b) Testing of new constituents other than proteins

The components analysed in MON 94313 were compositionally similar compared to conventional soybean. Therefore, no testing of any constituent other than the introduced proteins is required.

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[4] Codex Alimentarius, 2009. Foods derived from modern biotechnology. Codex Alimentarius, Second Edition, 1-85. – Accessed on 06 September 2022.
[5] CropLife International - http://www.biotradestatus.com/ - Accessed on 06 September 2022.
[6] Open EFSA - https://open.efsa.europa.eu/ - Accessed on 06 September 2022.

(c) Information on natural food or feed constituents

No relevant changes in the composition of MON 94313 were detected compared to conventional soybean. Therefore, the levels of food and feed constituents in MON 94313 have not been altered.

(d) Testing of the whole genetically modified food and feed

The data from molecular, compositional, agronomic and phenotypic testing demonstrate that MON 94313 does not display any biologically relevant differences from its conventional counterpart, except for the introduced herbicide tolerant traits. In addition, the safety for humans and animals of the MON 94313 DMO, PAT, FT_T.1 and TDO proteins has been demonstrated.

Based on this weight of evidence, no more data are required to demonstrate that MON 94313 is as safe as conventional soybean from a food and feed perspective and therefore it can be concluded that there was no evidence of any adverse effects on human or animal health.

Nonetheless, inspite of not being scientifically justified, in accordance with Commission Implementing Regulation (EU) No 503/2013, a 90-day feeding study with soybean grain from MON 94313 in rats was performed. As expected, the study supports the conclusion that MON 94313 is as safe as conventional soybean from a food and feed perspective.

6. ALLERGENICITY

(a) Assessment of allergenicity of the newly expressed proteins

The *dmo, pat, ft_t.1* and *TDO* genes are the only genes expressing novel proteins in MON 94313. Therefore, the safety assessment of the newly expressed proteins is focused on MON 94313 DMO, PAT, FT_T.1 and TDO proteins expressed in MON 94313.

Following the guidelines adopted by the Codex Alimentarius[4], an assessment of the allergenic potential of the newly expressed proteins was conducted and demonstrated that it is unlikely that the MON 94313 DMO, PAT, FT_T.1 and TDO proteins will cause allergenicity concerns due to the following considerations:

- The proteins are from non-allergenic sources;
- The proteins do not share structural similarities with known allergens; and
- The proteins are rapidly digested by proteases found in the human gastrointestinal tract (pepsin and pancreatin) under physiological conditions.

In addition, the lack of allergenic potential of the DMO and PAT proteins has previously been established on several occasions by several global regulatory agencies around the world[5] including the EFSA[6].

Based on this weight of evidence, no more data are required to demonstrate the safety of the newly expressed proteins in MON 94313.

(b) Assessment of allergenicity of the whole genetically modified plant

It has been demonstrated that MON 94313 has endogenous allergen levels similar to conventional soybeans and therefore is as safe as conventional soybeans in terms of allergenicity.

Therefore, MON 94313 and any food (or feed) derived from it, does not pose a significant allergenic risk to humans or animals..

7. NUTRITIONAL ASSESSMENT

(a) Nutritional assessment of the genetically modified food

Detailed compositional and nutritional comparisons of MON 94313, a conventional counterpart and commercially available reference soybean varieties confirmed that MON 94313 is compositionally not different from conventional soybean.

(b) Nutritional assessment of the genetically modified feed

See Section 7 (a).

8. EXPOSURE ASSESSMENT – ANTICIPATED INTAKE/EXTENT OF USE

The exposure assessment in humans and animals indicates that there is minimal dietary exposure to MON 94313 DMO, PAT, FT_T.1 and TDO proteins from the hypothetical consumption of foods and feed derived from MON 94313. No anticipated changes in the intake and/or extent of use of soybean or derived products for use as or in food or feed would be expected as a result of the addition of MON 94313 to the soybean supply. If ever commercialised, MON 94313 would replace a portion of current soybean such that the intake or use will represent some fraction of the total products derived from soybean.

9. RISK CHARACTERISATION

It has been demonstrated that MON 94313 is as safe as conventional soybean.

Molecular characterisation of MON 94313 did not raise any safety concern and did not show any evidence of unintended changes. Detailed compositional comparisons of MON 94313, its conventional counterpart and commercial reference varieties demonstrated that MON 94313 is compositionally similar to the conventional counterpart and that MON 94313 is not a contributor to compositional variability in soybean. The assessed phenotypic and agronomic characteristics of MON 94313 were within the range expected for soybean and did not show any phenotypic changes indicative of increased plant weed/pest potential of MON 94313 compared to conventional soybean. An extensive characterisation of the MON 94313 DMO, PAT, FT_T.1 and TDO proteins, confirmed that these proteins are safe for human and animal consumption. Additionally, the exposure assessment in humans and animals did not indicate any safety concerns.

In summary, there are no signs of adverse or unanticipated effects observed in a number of safety studies and the pre-market risk characterisation for food and feed use of MON 94313. If ever commercialised, the consumption of food and feed derived from MON 94313 would be as safe as the consumption of its conventional counterpart. It can be concluded that the food derived from MON 94313 is not nutritionally disadvantageous for the consumer compared to the food which it is intended to replace. Finally, it can be also concluded that the feed derived from MON 94313 does not harm or mislead the consumer by impairing distinctive features of the animal products compared to conventionally produced feed.

Based on the above, it is unlikely that MON 94313 will have an adverse effect on human and animal health and the environment, in the context of its intended hypothetical uses, which cover food and feed uses, import and processing as any other soybean.

Given the weight of evidence supporting the safety of MON 94313 and the negligible hazard of the newly expressed genetic elements, it is reasonable to conclude that the uncertainties associated with the risk characterisation are negligible. Therefore, specific risk management measures for MON 94313 in the EU are not considered necessary.

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10. POST-MARKET MONITORING ON THE GENETICALLY MODIFIED FOOD OR FEED

As demonstrated in this application, there are no intrinsic hazards related to MON 94313. No data have emerged to indicate that MON 94313 is less safe than its conventional counterpart. The pre-market risk characterisation for food and feed use of MON 94313 demonstrates that the risks of consumption of MON 94313 or its derived products are no different from the risks associated with the consumption of conventional soybean and soybean-derived products. As a consequence, specific risk management measures are not indicated and post-market monitoring of the use of this soybean, if ever commercialised, for food and feed is not considered necessary.

11. ENVIRONMENTAL ASSESSMENT

11.1. Mechanism of interaction between the genetically modified plant and target organisms

Not relevant given the scope of the application.

11.2. Potential changes in the interactions of the genetically modified plant with the biotic environment resulting from the genetic modification

The scope of this application covers the hypothetical import, processing and all uses as any other soybean, but excludes the cultivation of MON 94313 in the EU. Therefore, no deliberate release of viable plant material in the EU environment is expected, and interaction of MON 94313 with the biotic environment will be limited.

(a) Persistence and invasiveness

Results from the environmental assessment support the conclusion that the ability of MON 94313 to persist in agricultural fields or invade non-agricultural habitats is comparable to that of conventional soybean in the EU. Thus, MON 94313 is not more likely to represent an agronomic problem in agricultural fields or become more invasive in natural habitats and no adverse effects on ecological functions within agricultural production fields or on biodiversity is expected as a result of the hypothetical import, processing and all uses as any other soybean.

(b) Selective advantage or disadvantage

Compared with conventional soybean, the introduced herbicide tolerance traits in MON 94313 confer a selective advantage only under specific conditions (*i.e.* following treatment with dicamba- and/or glufosinate- and/or 2,4-D- and/or mesotrione- based herbicides), which is short in duration. This advantage is of purely agronomic interest and present negligible risk to the non-agricultural environments, because of the poor survival characteristics of soybean under most European conditions. Therefore, the likelihood is negligible for the introduced traits in MON 94313 to confer any meaningful competitive advantage or disadvantage of relevance to the environment.

(c) Potential for gene transfer

Given the low likelihood of occurrence of horizontal gene transfer and lack of adverse consequences if it were to occur, the hypothetical import, processing, and food and feed use of MON 94313 in the EU is not likely to adversely impact human, animal, or environmental health, and poses negligible risk.

(d) Interactions between the genetically modified plant and target organisms

Not relevant given the scope of the application.

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(e) Interactions of the genetically modified plant with non-target organisms

Given the low levels of environmental exposure combined with low hazard from exposure of MON 94313 to non-target organisms (NTOs), the likelihood of adverse effects to NTO communities that perform in-field ecological functions and NTO communities outside of the field from import of MON 94313, is negligible.

(f) Effects on human health

Given the low levels of environmental exposure combined with the negligible hazard occurring from the contact with MON 94313 grain, the likelihood for any adverse effects on humans and animals handling MON 94313 hypothetical import and processing in the EU is negligible.

(g) Effects on animal health

See Section 11.2.(f).

(h) Effects on biogeochemical processes

Given the low level of environmental exposure combined with a lack of hazard, the hypothetical import, processing and all uses of MON 94313 as any other soybean in the EU is not likely to adversely impact soil micro-organisms that perform ecological functions in-field or in non-agricultural habitats, and therefore poses negligible environmental risk.

(i) Impacts of the specific cultivation, management and harvesting techniques

Not relevant given the scope of the application.

11.3. Potential interactions with the abiotic environment

MON 94313 carry traits of agronomic interest: herbicide tolerance. As MON 94313 is shown not to be different from conventional soybean (with the exception of the introduced traits, imparted by the expression of the MON 94313 DMO, PAT, FT_T.1 and TDO proteins), with respect to its composition and agronomic and phenotypic characteristics, there is no evidence that this soybean would be any different from conventional soybean with regard to its baseline interactions with the abiotic environment.

Although the MON 94313 DMO, PAT, FT_T.1 and TDO proteins are expressed in soybean, they already have a safe history of use and they have no known negative interactions with the abiotic environment.

In addition, because this application is for hypothetical import, processing and all uses as any other soybean in the EU, but excludes cultivation, interaction of MON 94313 with the environment will be limited. Moreover, no negative impact of MON 94313 on the abiotic environment is expected to result from the import, processing and all uses as any other soybean in the EU.

11.4. Risk characterisation

Results from the environmental risk assessment which takes into consideration the risk characterisation and includes results described above addressing risk hypotheses for the specific areas of assessment laid down in EFSA guidance of 2010[7], support a conclusion that the hypothetical import, processing and all uses in the EU (excluding cultivation) of MON 94313, as any other soybean, would represent negligible risk to human and animal health and the environment, and would pose no greater risk than the import and processing of conventional soybean. Because no immediate adverse effects are expected, the probability of long-term adverse effects is also negligible.

[7] EFSA, 2010. Guidance on the environmental risk assessment of genetically modified plants. The EFSA Journal, 8(11):1879, 1-111. – Accessed on 06 September 2022

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12. ENVIRONMENTAL MONITORING PLAN

(a) General (risk assessment, background information)

As required by Article 5(5)(b) and 17(5)(b) of Regulation (EC) No 1829/2003 the proposed Post-Market Environmental Monitoring (PMEM) plan for MON 94313 has been developed according to the principles and objectives outlined in Annex VII of Directive 2001/18/EC and Decision 2002/811/EC establishing guidance notes supplementing Annex VII to Directive 2001/18/EC. The PMEM also takes into account the Scientific Opinion on guidance on the Post-Market Environmental Monitoring of genetically modified plants[8].

(b) Interplay between environmental risk assessment and monitoring

The scope of this application is the authorisation of MON 94313 for hypothetical import, processing, food and feed use in the European Union (EU) under Regulation (EC) No 1829/2003. The scope of the application does not include authorisation for the cultivation of MON 94313 in the EU.

An environmental risk assessment (e.r.a.) was carried out for MON 94313 according to the principles laid down in Annex II to Directive 2001/18/EC and Decision 2002/623/EC establishing guidance notes supplementing Annex II to Directive 2001/18/EC. The scientific evaluation of the characteristics of MON 94313 in the e.r.a. (Section 5 of Part II – Scientific information) has shown that the risk for potential adverse effects on human and animal health or the environment is negligible in the context of the intended uses of MON 94313.

(c) Case-specific genetically modified plant monitoring (approach, strategy, method and analysis)

The scientific evaluation of the characteristics of MON 94313 in the e.r.a. has shown that the risk for potential adverse effects on human and animal health or the environment is negligible in the context of the intended uses of MON 94313. It is therefore considered that there is no need for case-specific monitoring.

(d) General surveillance of the impact of the genetically modified plant (approach, strategy, method and analysis)

Any potential adverse effects of MON 94313 on human health and the environment, which were not anticipated in the e.r.a., can be addressed under the general surveillance. General surveillance is largely based on routine observation and implies the collection, scientific evaluation and reporting of reliable scientific evidence, in order to be able to identify whether unanticipated, direct or indirect, immediate or delayed adverse effects have been caused by the placing on the market of a GM crop in its receiving environment.

In order to allow detection of the broadest possible scope of unanticipated adverse effects, general surveillance is performed by either selected, existing networks, or by specific company stewardship programmes, or by a combination of both. The consent holder will ensure that appropriate technical information on MON 94313 and relevant legislation will be available for the relevant networks, in addition to further relevant information from a number of sources, including industry and government websites, official registers and government publications.

[8] EFSA, 2011. Guidance on the post-market environmental monitoring (PMEM) of genetically modified plants. The EFSA Journal, 9 (8):2316, 1-40. – Accessed on 06 September 2022

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Following the approval of these soybean in the EU, the consent holder will approach key stakeholders and key networks of stakeholders of the products (including international grain traders, soybean processors and users of soybean grain for animal feed) and inform them that the products have been authorised. The consent holder will request key stakeholders and networks for their participation in the general surveillance of the placing on the market of these soybean, in accordance with the provisions of Directive 2001/18/EC and the consent. Key stakeholders and networks will be requested to be aware of the use of these soybean and to inform the consent holder in case of potential occurrence of any unanticipated adverse effects to health or the environment, which they might attribute to the import or use of these products. Appropriate technical information on MON 94313 will be provided to them.

Where there is scientifically valid evidence of a potential adverse effect (whether direct or indirect), linked to the genetic modification, then further evaluation of the consequence of that effect should be science-based and compared with available baseline information. Relevant baseline information will reflect prevalent use practices and the associated impact of these practices on the environment. Where scientific evaluation of the observation confirms the possibility of an unanticipated adverse effect, this would be investigated further to establish a correlation, if present, between the use of MON 94313 and the observed effect. The evaluation should consider the consequence of the observed effect and remedial action, if necessary, should be proportionate to the significance of the observed effect.

(e) Reporting the results of monitoring

In accordance with Regulation (EC) No 1829/2003, the authorisation holder is responsible to inform the European Commission of the results of the general surveillance.

If information that confirms an adverse effect of MON 94313 and that alters the existing risk assessment becomes available, the authorisation holder will immediately investigate and inform the European Commission. The authorisation holder, in collaboration with the European Commission and based on a scientific evaluation of the potential consequences of the observed adverse effect, will define and implement management measures to protect human and animal health or the environment, as necessary. It is important that the remedial action is proportionate to the significance of the confirmed effect.

The authorisation holder will submit an annual monitoring report including results of the general surveillance in accordance with the conditions of the authorisation. The report will contain information on unanticipated adverse effects, if any, that have arisen from handling and use of viable MON 94313.

The report will include a scientific evaluation of the confirmed adverse effect, a conclusion of the safety of MON 94313 and, as appropriate, the measures that were taken to ensure the safety of human and animal health or the environment.

The report will also clearly state which parts of the provided information are considered confidential, together with a verifiable justification for confidentiality in accordance with Article 30 of Regulation (EC) No 1829/2003. Confidential parts of such report shall be submitted in separate documents.

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13. DETECTION AND EVENT-SPECIFIC IDENTIFICATION TECHNIQUES FOR THE GENETICALLY MODIFIED PLANT

The presence of the *dmo, pat, ft_t.1* and *TDO* genes and the MON 94313 DMO, PAT, FT_T.1 and TDO proteins can be identified by employing different techniques. PCR can identify the inserted nucleotide sequence, while the newly expressed proteins can be detected, by optimised tissue extraction, standardised electrophoretic blotting and immunodetection methodologies. A specific PCR-based assay allowing the identification and quantification of MON 94313 has been provided to the European Union Reference Laboratory for GM Food and Feed (EU-RL-GMFF), Joint Research Centre (JRC)[9].

14. INFORMATION RELATING TO PREVIOUS RELEASES OF THE GENETICALLY MODIFIED PLANT (FOR ENVIRONMENTAL SAFETY ASPECTS)

14.1. History of previous releases of the genetically modified plant notified under Part B of Directive 2001/18/EC or under Part B of Directive 90/220/EEC by the same notifier

(a) Notification number

There is no history of release of MON 94313 in the EU.

(b) Conclusions of post-release monitoring

Not applicable

(c) Results of the release in respect to any risk to human health and the environment, submitted to the competent authority according to Article 10 of Directive 2001/18/EC)

Not applicable

14.2. History of previous releases of the genetically modified plant carried out outside the Union by the same notifier

(a) Release country

MON 94313 has been field tested in the US since 2020, Argentina since 2021, Chile since 2021 and Brazil in 2022.

(b) Authority overseeing the release

US: United States Department of Agriculture (USDA).

Argentina: MAGyP (Ministerio de Agricultura, Ganadería y Pesca) - Ministry of Agriculture, Livestock and Fisheries

Chile: Secretariat of Agriculture and Livestock (SAG)

Brazil: Comissão Técnica Nacional de Biossegurança (CTNBio) - National Biosafety Technical Commission.

(c) Release site

In major soybean growing regions.

[9] European Union Reference Laboratory for GM Food and Feed, Joint Research Centre: http://gmo-crl.jrc.ec.europa.eu/ - Accessed on 06 September 2022.

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(d) Aim of the release

US: Regulatory, efficacy, yield testings, breeding, product development, demonstration trials and nurseries.

Argentina: Regulatory.

Chile: nurseries.

Brazil: Regulatory and nurseries.

(e) Duration of the release

US: 2020-2022.

Argentina: 2021-2022.

Chile: 2021-2022.

Brazil: 2022.

(f) Aim of post-releases monitoring

Assessment of volunteers.

(g) Duration of post-releases monitoring

12 months.

(h) Conclusions of post-release monitoring

US, Argentina, Chile: In general, no volunteers have been observed since soybean is an annual crop and seed are very susceptible to overwintering conditions. If volunteers occur, practice is to eliminate them manually or chemically to prevent occurrence in subsequent crops.

Brazil: Plants are currently in the field (2022) so post-release monitoring is not completed yet.

(i) Results of the release with respect to any risk to human health and the environment

Field-testing provided no evidence that MON 94313 or derived products would be the cause of any adverse effects to human health or to the environment.

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EXHIBIT J

Application for authorisation to place on the market MON 94313 soybean in the European Union, according to Regulation (EC) No 1829/2003 on genetically modified food and feed

Part II

Scientific information

Hazard identification and characterisation: Molecular characterisation

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Data protection.
This application contains scientific data and other information which are protected in accordance with Art. 31 of Regulation (EC) No 1829/2003.

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LIST OF ABBREVIATIONS AND ACRONYMS

NOTE: ***ABBREVIATIONS AND ACRONYMS ARE USED THROUGHOUT THE ENTIRE APPLICATION. THESE ARE DESIGNED TO TAKE UP LESS SPACE AND MAKE THE WRITING EASIER TO READ. FULL NAMES ARE SPECIFIED WHEN FIRST TIME MENTIONED IN THE TEXT.***

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NOTE: ***THE FOLLOWING APPENDICES ARE PROVIDED UNDER THE "LIST OF ANNEXES, REFERENCES AND CHECKLIST" SECTION OF THIS APPLICATION:***

- ***APPENDIX 1_AN OVERVIEW TABLE OF ALL STUDIES CARRIED OUT IN SUPPORT TO THIS APPLICATION FOR AUTHORISATION OF MON 94313 SOYBEAN (HEREAFTER REFERRED TO AS MON 94313),***
- ***APPENDIX 2_A SYNOPTIC TABLE OF THE APPLICATION,***
- ***APPENDIX 3_AN OVERVIEW TABLE SUMMARISING THE ALGORITHMS AND DATABASES USED IN THE BIOINFORMATICS ANALYSES PERFORMED WITH MON 94313,***
- ***APPENDIX 4_A SUMMARY TABLE WITH INFORMATION REGARDING THE FIELD TRIAL RELATED TO THE NEWLY EXPRESSED PROTEIN LEVELS AND***
- ***APPENDIX 5_STAGED APPROACH ON ENVIRONMENTAL RISK ASSESSMENT (e.r.a.) TO ADDRESS THE POTENTIAL HARM OF THE INTRODUCED TRAITS IN MON 94313 SOYBEAN.***

APPENDICES C TO E (SCHEMATIC SUMMARIES FOR INSECT RESISTANCE MANAGEMENT, NTO STUDIES, AND STATISTICAL DESIGN AND ANALYSIS FOR EACH E.R.A.-RELATED STUDY, RESPECTIVELY) ARE NOT RELEVANT TO THIS APPLICATION. HENCE, THEY ARE NOT PROVIDED.

THE DMO AND PAT PROTEINS HAVE BEEN ASSESSED BY SEVERAL GLOBAL REGULATORY AGENCIES[1] INCLUDING THE EFSA[2]. ALSO, FT.T.1 HAS BEEN ASSESSED BY GLOBAL REGULATORY AGENCIES[1] AND IS BEING ASSESSED BY THE EFSA[2].

FINALLY, MON 94313 SINGLE PRODUCT IS NOT AND WILL NOT BE COMMERCIALISED ON ITS OWN, INSTEAD STACKED PRODUCTS, MADE VIA TRADITIONAL BREEDING, ARE THE AIMED COMMERCIAL PRODUCTS. THEREFORE, THE HYPOTHETICAL IMPORT OF THIS PRODUCT IN THE EU IS CONSIDERED IN THIS APPLICATION.

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[1] CropLife International - http://www.biotradestatus.com/ - Accessed on 04 August 2022.

[2] Open EFSA - https://open.efsa.europa.eu/ - Accessed on 04 August 2022.

1.2.Molecular characterisation

1.2.1. Information relating to the genetic modification

1.2.1.1. *Description of the methods used for the genetic modification*

a) Method of genetic transformation including relevant references

MON 94313 was developed through *Agrobacterium*-mediated transformation of soybean, based on the methods described by Ye *et al.* (2008). After co-culturing with *Agrobacterium* AB30 strain carrying the plasmid vector, meristem explants were placed on selection medium containing spectinomycin to select for transgenic events and carbenicillin, cefotaxime and timentin to inhibit the overgrowth of *Agrobacterium*. The putative transgenic plants (R0) with normal phenotypic characteristics were plugged and screened using copy number assay and linkage analysis. Events which passed the advancement criteria (such as a single copy of the insert unlinked with the selectable marker, no presence of vector backbone, no insertion into repetitive regions or gene sequences) were selected and transferred to soil for growth and further assessment.

The R0 plants generated through the transformation process described above were self-pollinated to produce R1 seed, and the unlinked insertions of T-DNA I (containing the expression cassettes) and T-DNA II (containing the selectable marker gene) were segregated. The *splA* scorable phenotype and *aadA* coding sequence polymerase chain reaction (PCR) analyses were used to eliminate any seeds or plants from further development that contain T-DNA II. Subsequently, R1 plants homozygous for T-DNA I were selected for further development and their progenies were subjected to further molecular analysis, herbicide tolerance/efficacy testing and phenotypic assessments. As is typical of a commercial event production and selection process, hundreds of different transformation events (regenerants) were generated in the laboratory using PV-GMHT529103. After careful selection and evaluation of these events in the laboratory, greenhouse and field, MON 94313 was selected as the lead event based on superior agronomic, phenotypic, and molecular characteristics. Studies on MON 94313 were initiated to further characterise the genetic insertion and the expressed products, and to establish the food and feed safety and unaltered environmental risk compared to commercial soybean. The major development steps of MON 94313 are depicted in **Figure 1**.

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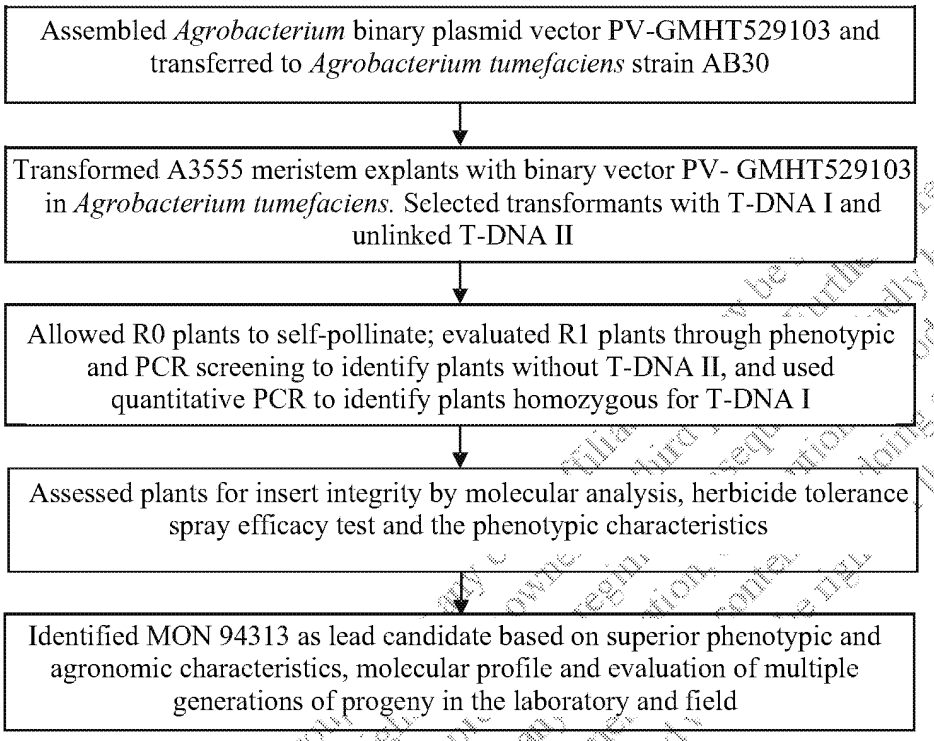


Figure 1. Schematic development of MON 94313

b) Recipient plant material

The conventional soybean variety A3555, used as the recipient for the *dmo, pat, ft_t.1* and *TDO* expression cassettes that produced MON 94313, was developed by Monsanto Company.

c) Species and strain of *Agrobacterium* and other microbes, if used for the genetic transformation process

The organism used for genetic transformation of soybean to produce MON 94313 was *Agrobacterium tumefaciens* strain AB30.

d) Helper plasmids, if used during the genetic transformation process

No helper plasmid was used in the transformation process.

e) Source of carrier nucleic acid(s), if used during the genetic transformation process

No carrier DNA was used during the transformation process.

1.2.1.2. *Nature and source of vector used*

a) A physical map of the functional elements and other plasmid/vector components together with the relevant information needed for the interpretation of the molecular analyses

A circular map of plasmid vector PV-GMHT529103 is presented in M-815764-02-1 (2022) This physical map contains relevant information needed for the interpretation of the molecular analysis.

b) Table identifying each component of the plasmid/vector (including the region intended for insertion), its size, its origin and its intended function.

A description of the genetic elements in PV-GMHT529103 is provided in M-815764-02-1 (2022).

1.2.1.3. *Source of donor nucleic acid(s) used for transformation, size and intended function of each constituent fragment of the region intended for insertion*

a) Complete sequence of the nucleic acid(s) intended to be inserted, including information on any deliberate alteration(s) to the corresponding sequence(s) in the donor organism(s)

PV-GMHT529103 contains two T-DNAs (transfer DNA) which are each delineated by Right and Left Border regions and the vector backbone sequences. T-DNA I is approximately 10.6 kb and contains the *dmo*, *pat*, *ft_t.1*, and *TDO* expression cassettes. T-DNA II is approximately 7.1 kb and contains the *splA* and *aadA* expression cassettes (selectable markers). The vector backbone sequence is approximately 6.8 kb and contains the *nptII* expression cassette. Following transformation, segregation, selection and screening were used to isolate those plants that contain the T-DNA I sequence with the *dmo, pat, ft_t.1,* and *TDO* expression cassettes but not the T-DNA II or plasmid backbone sequences.

For detailed descriptions about the genetic elements, their positions, orientations, sizes, intended functions and origins, *see* M-815764-02-1 (2022).

b) History of safe use of the gene product(s) arising from the regions intended for insertion

The gene products arising from the regions intended for insertion in MON 94313 are the herbicidal proteins DMO, PAT, FT_T.1 and TDO. Their respective deduced full-length amino acid sequences are shown in **Figure 2** to **Figure 5**.

DMO protein

DMO protein, isolated from *Stenotrophomonas maltophilia* (Herman *et al.*, 2005; Krueger *et al.*, 1989; Palleroni and Bradbury, 1993), has a demonstrated long history of safe use and the DMO protein expressed in other crop products has been assessed by several global regulatory agencies[13] including the EFSA[14]. The safety of DMO proteins has also been well established in the scientific literature based on protein safety across various genetically modified (GM) crops (Wang *et al.*, 2016). DMO is an enzyme that catalyses the demethylation of dicamba to the non-herbicidal compounds 3,6-dichlorosalicylic acid (DCSA) and formaldehyde

[13] CropLife International - http://www.biotradestatus.com/ - Accessed on 04 August 2022.

[14] Open EFSA - https://open.efsa.europa.eu/ - Accessed on 04 August 2022.

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(Chakraborty *et al.*, 2005). DCSA is a known metabolite of dicamba in cotton, soybean, soil, and livestock, whose safety has been evaluated by the FAO-WHO[15] and EPA[16]. The other reaction product, formaldehyde, is found naturally in many plants (Adrian-Romero *et al.*, 1999).

There is a wide variety of oxygenases present in bacteria and plants currently widely prevalent in the environment and consumed by humans or animals (Ferraro *et al.*, 2005; Schmidt and Shaw, 2001), establishing that animals and humans are extensively exposed to these types of enzymes with no adverse effects noted.

```
  1 MATATTTATA AFSGVVSVGT ETRRIYSFSH LQPSAAFPAK PSSFKSLKLK
 51 QSARLTRRLD HRPFVVRCML TFVRNAWYVA ALPEELSEKP LGRTILDTPL
101 ALYRQPDGVV AALLDICPHR FAPLSDGILV NGHLQCPYHG LEFDGGGQCV
151 HNPHGNGARP ASLNVRSFPV VERDALIWIW PGDPALADPG AIPDFGCRVD
201 PAYRTVGGYG HVDCNYKLLV DNLMDLGHAQ YVHRANAQTD AFDRLEREVI
251 VGDGEIQALM KIPGGTPSVL MAKFLRGANT PVDAWNDIRW NKVSAMLNFI
301 AVAPEGTPKE QSIHSRGTHI LTPETEASCH YFFGSSRNFG IDDPEMDGVL
351 RSWQAQALVK EDKVVVEAIE RRRAYVEANG IRPAMLSCDE AAVRVSREIE
401 KLEQLEAA
```

Figure 2. Deduced amino acid sequence of APG6 chloroplast targeting sequence and the MON 94313 DMO protein

The amino acid sequence of the MON 94313 DMO precursor protein was deduced from the full-length coding nucleotide sequence present in PV-GMHT529103. The first 68 amino acids of the precursor protein (underlined) are the CTP from *APG6* gene. The CTP targets MON 94313 DMO precursor protein to the chloroplast and is cleaved in the chloroplast producing the mature 340 amino acid DMO proteins that begin with the methionine at position 69.

PAT protein

The *pat* gene is derived from the bacterium *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988). *Streptomyces* species are widespread in the environment and present no known allergenic or toxicity issues (Kutzner, 1981; Kampfer, 2006), though human exposure is quite common (Goodfellow and Williams, 1983). *S. viridochromogenes* is not considered pathogenic to plants, humans or other animals (Locci, 1989; Cross, 1989; Goodfellow and Williams, 1983). *S. viridochromogenes* is widespread in the environment and the history of safe use is discussed in Hérouet *et al.* (2005). This organism has been extensively reviewed by several global regulatory agencies[13] including the EFSA[14].

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[15] FAO-WHO, 2021 - Summary report: Acceptable daily intakes, acute reference doses, residue definitions, recommended maximum residue limits,supervised trials median residue values recorded and other values recorded. Agriculture Organization of the United Nations, World Health Organization. - Accessed on 04 August 2022.

[16] EPA, 2006 - Reregistration eligibility decision for dicamba and associated salts. U.S. Environmental Protection Agency, Washington, D.C – Accessed on 04 August 2022.

```
  1 MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL
 51 ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
101 GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
151 AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI
```

Figure 3. Deduced amino acid sequence of the PAT protein

The amino acid sequence of the MON 94313 PAT protein was deduced from the full-length coding nucleotide sequence present in PV-GMHT529103. The lead methionine (boxed) of the PAT protein produced in MON 94313 is cleaved during a co-translational process in MON 94313.

FT_T.1 protein

The *ft_t.1* gene is derived from the bacterium *Sphingobium herbicidovorans*. *S. herbicidovorans* is a common gram-negative, rod-shaped, non-motile, non-spore-forming soil bacterium (Zipper *et al.*, 1996; Takeuchi *et al.*, 2001), which is strictly aerobic and chemo-organotrophic, and not known to be associated with human disease. Members of the genus *Sphingobium* have been isolated from a wide variety of habitats including soil and freshwater (Chaudhary *et al.*, 2017). *Sphingobium* species have been isolated from food products such as corn (Rijavec *et al.*, 2007), papaya (Schroder *et al.*, 2007) and tomato (Enya *et al.*, 2006). The biosysthesis and biodegrading properties of this genus have been exploited in the food industry (Pozo *et al.*, 2007; Fialho *et al.*, 2008), bioremediation (Jin *et al.*, 2013; Alarcon *et al.*, 2008), and biofuel industry (Varman *et al.*, 2016). The ubiquitous presence of *Sphingobium* species in the environment has resulted in widespread human and animal exposure without any known adverse safety or allergenicity reports. *S. herbicidovorans* history of safe use has been extensively reviewed by several global regulatory agencies[13] including the EFSA[14].

```
1   MHAALTPLTN KYRFIDVQPL TGVLGAEITG VDLREPLDDS TWNEILDAFH
51  TYQVIYFPGQ AITNEQHIAF SRRFGPVDPV PILKSIEGYP EVQMIRREAN
101 ESSRYIGDDW HADSTFLDAP PAAVVMRAIE VPEYGGDTGF LSMYSAWETL
151 SPTMQATIEG LNVVHSATKV FGSLYQATNW RFSNTSVKVM DVDAGDRETV
201 HPLVVTHPVT GRRALYCNQV YCQKIQGMTD AESKSLLQFL YEHATQFDFT
251 CRVRWKKDQV LVWDNLCTMH RAVPDYAGKF RYLTRTTVAG DKPSR
```

Figure 4. Deduced amino acid sequence of the FT_T.1 protein

The amino acid sequence of the MON 94313 FT_T.1 protein was deduced from the full-length coding nucleotide sequence present in PV-GMHT529103. FT_T.1 protein is expressed as a mature 295 amino acid FT_T.1 protein that begins with the lead methionine

TDO protein

The *TDO* gene comes from *Oryza sativa*[17], a crop plant with long history of exposure in food or feed. Asian cultivated rice (*O. sativa*) is one of the most important crops in the world serving as a primary food source for more than half of the world's population (Khush, 1997). Rice is consumed in various forms such as brown, milled, polished and parboiled which are the major rice products consumed by humans in the form of grain after being cooked. Rice is also

[17] OECD, 2021 - Revised Consensus Document on the Biology of Rice (*Oryza sativa* L.). OECD, ENV/CBC/MONO(2021)42, 89. – Accessed on 04 August 2022.

COR-AAD1-00007181

consumed as food ingredients which are part of food products. The primary nutrients provided by rice are carbohydrates and proteins. Rice is also widely used as feed for livestock. This is fed in various forms such rice grain, hulls, bran, straw, polishings, and whole crop silage[18].

Generally, rice is considered to be a safe source of food and feed and is not considered by allergists to be a common source of allergens. There are very few compounds in rice which are considered unfavourable for human or animal food/feed, and these compounds have not been observed to exist at levels in rice-based foods that would be a concern for food or feed safety[18].

TDO protein shares the common high order structure of Fe (II)/alpha-ketoglutarate-dependent dioxygenases that contains a classical dioxygenase active pocket including an iron atom coordinated by two histidine residues and one aspartic acid or glutamate residue (Hausinger, 2004). These alpha-ketoglutarate-dependent dioxygenases have been identified in a broad range of organisms including bacteria, fungi, plants, and vertebrates, which have been extensively consumed by both humans and animals (Hausinger, 2004; Kundu, 2012) without any reports of adverse effects. TDO-like proteins with a high level of similarity are also found in other plant species, most especially in monocot species, including cultivated species such as wheat and maize, and more distantly in dicot species (Maeda and Dudareva, 2012). Therefore, both TDO and proteins with structural homology to TDO are present in plants commonly consumed as food and feed, and the presence of TDO in soybean does not represent a new exposure of this protein in the human or animal diet.

```
  1 MADESWRAPA IVQELAAAGV EEPPSRYLLR EKDRSDVKLV AAELPEPLPV
 51 VDLSRLDGAE EATKLRVALQ NWGFFLLTNH GVEASLMDSV MNLSREFFNQ
101 PIERKQKFSN LIDGKNFQLQ GYGTDRVVTQ DQILDWSDRL HLRVEPKEEQ
151 DLAFWPDHPE SFRDVLNKYA SGTKRIRDDI IQAMAKLLEL DEDYFLDRLN
201 EAPAFARFNY YPPCPRPDLV FGIRPHSDGT LLTILLVDKD VSGLQVQRDG
251 KWSNVEATPH TLLINLGDTM EVMCNGIFRS PVHRVVTNAE KERISLAMLY
301 SVNDEKDIEP AAGLLDENRP ARYRKVSVEE FRAGIFGKFS RGERYIDSLR
351 I
```

Figure 5. Deduced amino acid sequence of the TDO protein

The amino acid sequence of the MON 94313 TDO protein was deduced from the full-length coding nucleotide sequence present in PV-GMHT529103. The lead methionine (boxed with solid line) of the TDO protein produced in MON 94313 is cleaved during a co-translational process in MON 94313.

In addition to *dmo, pat, ft_t.1* and *TDO*, the associated regulatory genetic elements were obtained from the following sources: *Agrobacterium tumefaciens, Arabidopsis thaliana, Medicago truncatula* and *Zea mays.* that have an extensive history of safe use.

There is no evidence of human or animal toxicity or allergenicity for the donor organisms of the coding and non-coding DNA sequences present in MON 94313. DNA has always been present in food and, upon consumption, is quickly degraded by restriction nucleases present in the gastrointestinal tract of humans and animals.

[18] OECD, 2016 - Revised Consensus Document on compositional considerations for new varieties of rice (Oryza sativa): key food and feed nutrients, anti-nutrients and other constituents – Accessed on 04 August 2022.

According to the US Food and Drug Administration[19], nucleic acids are present in the cells of every living organism, do not raise concerns as a component of food, and are generally recognised as safe (GRAS). Result from an International Lifescience Institute (ILSI)[20] workshop on safety considerations of DNA in food were reported (Jonas *et al.*, 2001) and confirmed that i) all DNA including recombinant DNA is composed of the same four nucleotides, ii) there are no changes to the chemical characteristics or the susceptibility to degradation by chemical or enzymatic hydrolysis of recombinant DNA as compared to non-recombinant DNA and iii) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome. Additionally, the EFSA has reported that a large number of experimental studies have shown that recombinant DNA consumed by livestock has not been subsequently detected in tissues, fluids or edible products of these farm animals[21].

In conclusion, the donor organisms and all DNA components inserted in MON 94313 are well understood and there is no evidence of any biosafety issues for any of the sequences that have been used.

c) Data on the possible relationship of the gene products with known toxins, anti-nutrients and allergens

The DMO, PAT, FT_T.1 and TDO protein sequences were assessed for their allergenicity, toxicity, and potential adverse biological activity.

Sequence similarities towards allergens were assessed using the FASTA sequence alignment and a sliding window search of the AD_2021 and FCD_2021 allergen databases while sequence similarities towards toxins and biologically active proteins were assessed using the FASTA sequence alignment search of the TOX_2021 and PRT_2021 databases, respectively.

For detailed descriptions about the assembly of the databases and the latest assessment of sequence similarities, *see* TRR0000689 (2021),TRR0000842 (2021), TRR0001380 (2022) and TRR0001385 (2022), respectively. The results of previous assessment for PAT, cited in TRR0000842 (2021) can be found in M-787376-01-1 (2020).

An overview table summarising the algorithms and databases used in the bioinformatics analyses performed with the MON 94313 sequences is included in **Appendix 3**.

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[19] US FDA, 1992. Statement of Policy: Foods derived from New Plant Varieties; Notice. Federal Register, 57, 22984-23005. – Accessed on 04 August 2022.

[20] On 1st May 2020, the International Life Sciences Institute (ILSI) changed its name to Agriculture and Food Systems Institute (AFSI).

[21] EFSA, 2007. Statement of EFSA on the fate of recombinant DNA or proteins in meat, milk and eggs from animals fed with GM feed. European Food Safety Authority, 1-8. – Accessed on 04 August 2022.

The results from the bioinformatic analyses indicated that no biologically relevant sequence similarities were observed between the DMO, PAT, FT_T.1 and TDO protein sequences and the allergens or biologically active proteins associated with adverse effects for human or animal health (TRR0001380, 2022; TRR0000842, 2021; TRR0001385, 2022). The results of previous assessment for PAT, cited in TRR0000842 (2021) can be found in M-787376-01-1 (2020).

d) Taxonomic classification of the donor organisms

MON 94313 was developed through *Agrobacterium*-mediated transformation of soybean, based on the methods described by Ye *et al.* (2008) as described in Section 1.2.1.1.

DMO donor organism: *Stenotrophomonas maltophilia*

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Xanthomonadales
Family: Xanthomonadaceae
Genus: *Stenotrophomonas*

PAT donor organism: *Streptomyces viridochromogenes*

Kingdom: Bacteria
Phylum: Actinobacteria
Class: Actinobacteria
Order: Actinomycetales
Family: Streptomycetaceae
Genus: *Streptomyces*

FT_T.1 donor organism: *Sphingobium herbicidovorans*

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Sphingomonadales
Family: Sphingomonadaceae
Genus: *Sphingobium*

TDO donor organism: *Oryza sativa*

Kingdom: Plantae
Phylum: Magnoliophyta
Class: Liliopsida
Order: Cyperales
Family: Poaceae
Genus: *Oryza*

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e) History of use of the donor organisms regarding food and feed safety

The *dmo* gene is derived from the bacterium *S. maltophilia* (Herman *et al.*, 2005; Palleroni and Bradbury, 1993). *S. maltophilia* is ubiquitous in the environment (Berg *et al.*, 1999; Echemendia, 2010; Ryan *et al.*, 2009; Qureshi *et al.*, 2005; Denton *et al.*, 1998; Denton and Kerr, 1998; Mukherjee and Roy, 2016), including in water and dairy products (An and Berg, 2018; Okuno *et al.*, 2018; Todaro *et al.*, 2011). These bacteria have been used as effective biocontrol agents in plant and animal pathogenesis (Mukherjee and Roy, 2016), and have antibacterial activity against both gram-positive and gram-negative bacteria (Dong *et al.*, 2015). *S. maltophilia* can be found in healthy individuals without causing any harm to human health (Denton and Kerr, 1998; Cunha, 2009; Heller *et al.*, 2016; Lira *et al.*, 2017), although these bacteria can form biofilms that become resistant to antibiotics (Berg and Martinez, 2015; Brooke *et al.*, 2017). The opportunistic pathogenicity of *S. maltophilia* is mainly associated with hosts with compromised immune systems rather than with any specific virulence genes of these bacteria. Thus, documented occurrences of *S. maltophilia* infections have been limited to immune-compromised individuals in hospital settings (Lira *et al.*, 2017). Other than the potential to become an opportunist pathogen in immune-compromised hosts, *S. maltophilia* is not known for human or animal pathogenicity. The history of safe use of *S. maltophilia* has been previously reviewed by several global regulatory agencies[13] including the EFSA[14] and no concerns on safety or allergenicity issues were identified.

The *pat* gene is derived from the bacterium *S. viridochromogenes* (Wohlleben *et al.*, 1988). The ubiquitous presence of *S. viridochromogenes* in the environment, the widespread human exposure without any adverse safety or allergenicity reports, and the successful reviews of several *pat*-containing events in various GM crops by several global regulatory agencies[13] including the EFSA[14] have identified no safety or allergenicity issues which further establish the safety of the donor organism.

The *ft_t.1* gene is a modified version of the *RdpA* gene derived from the bacterium *S. herbicidovorans* (Müller *et al.*, 2006). *S. herbicidovorans* is a common gram-negative, rod-shaped, non-motile, non-spore-forming soil bacterium (Zipper *et al.*, 1996; Takeuchi *et al.*, 2001), which is strictly aerobic and chemo-organotrophic, and not known to be associated with human disease. Members of the genus *Sphingobium* have been isolated from a wide variety of habitats including soil and freshwater (Chaudhary *et al.*, 2017). *Sphingobium* species have also been isolated from food products such as corn (Rijavec *et al.*, 2007), papaya (Thomas *et al.*, 2007) and tomato (Enya *et al.*, 2006). The biosysthesis and biodegrading properties of this genus have been exploited in the food industry (Pozo *et al.*, 2007; Fialho *et al.*, 2008), bioremediation (Jin *et al.*, 2013; Alarcon *et al.*, 2008), and biofuel industry (Varman *et al.*, 2016). The ubiquitous presence of *Sphingobium* species in the environment has resulted in widespread human and animal exposure without any known adverse safety or allergenicity reports. The history of safe use of *S. herbicidovorans* has been previously reviewed by several global regulatory agencies[13] and no concerns on safety or allergenicity issues were identified.

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The *TDO* gene comes from *Oryza sativa*[17], a crop plant with long history of exposure in food or feed. Asian cultivated rice (*O. sativa*) is one of the most important crops in the world serving as a primary food source for more than half of the world's population (Khush, 1997). Rice is cultivated in more than 80 countries around the world, being one of three major staple crops after corn and with a total production similar to wheat. *O. sativa* has two subspecies, *indica* and *japonica*, which account for almost all global rice production. Brown, milled, polished and parboiled rice are the major rice products consumed by humans in the form of grain after being cooked. Rice is also consumed as food ingredients which are part of food products. For example, rice flour is used in cereals, baby food, and snacks. The primary nutrients provided by rice are carbohydrates and proteins. Rice and rice by-products (hulls, polishings, straw) are also widely used as feed for livestock.

1.2.2. Information relating to the genetically modified plant

1.2.2.1. *General description of the trait(s) and characteristics which have been introduced or modified*

Herbicide tolerant traits

MON 94313 soybean was developed through *Agrobacterium tumefaciens* mediated transformation of soybean tissues to express MON 94313 DMO, PAT, FT_T.1 and TDO proteins conferring tolerance to dicamba-, glufosinate-, 2,4-D- and mesotrione-based herbicides, respectively. The combination of the dicamba, glufosinate, 2,4-D and mesotrione herbicides' distinct mechanisms-of-action provides an effective weed management system.

DMO protein

MON 94313 soybean contains a *dmo* gene from *S. maltophilia* that expresses a DMO protein (**Figure 2**) to confer tolerance to dicamba herbicide. The DMO protein is a mono-oxygenase that catalyses the demethylation of the broadleaf herbicide dicamba to the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) and formaldehyde, thus conferring dicamba tolerance (Chakraborty *et al.*, 2005). The DCSA product of the reaction catalysed by the DMO protein is a known metabolite of dicamba in conventional cotton, soybean, soil, and livestock, and its safety has been evaluated by the FAO-WHO[15] and EPA[16]. The safety of these proteins has also been reviewed and approved by several global regulatory agencies[13] including the EFSA[14]. The other reaction product, formaldehyde, is found naturally in many plants and edible fungi at levels up to several hundred ppm (Adrian-Romero *et al.*, 1999; Tashkov, 1996).

MON 94313 DMO is targeted to chloroplasts by a chloroplast transit peptide (CTP) to allow co-localisation with the endogenous reductase and ferredoxin enzymes that supply electrons for the DMO demethylation reaction as described by Behrens *et al.* (2007). In the construction of the plasmid vector used in the development of MON 94313, PV-GMHT529103, the *Albino and pale green 6* (*APG6)* chloroplast transit peptide coding sequence from *Arabidopsis thaliana* was joined to the *dmo* coding sequence; this coding sequence results in the production of a precursor protein consisting of the DMO protein and a N-terminal 68 amino acid chloroplast transit peptide APG6, which is utilised to target the precursor protein to the chloroplast (Herrmann, 1995; Klee *et al.*, 1987). Typically, transit peptides are precisely removed from the precursor protein following delivery to the targeted plastid (Della-Cioppa *et*

COR-AAD1-00007186

al., 1986) resulting in the full length protein. However, there are examples in the literature of alternatively processed forms of a protein targeted to a plant's chloroplast (Behrens *et al.*, 2007; Clark and Lamppa, 1992). Data from N-terminal sequencing analysis of the MON 94313-produced DMO indicate that processing of the DMO precursor protein expressed in MON 94313 produced a single isoform of the mature MON 94313 DMO protein with no additional N-terminal residues remaining from partial processing of the of the APG6 transit peptide.

Except for the additional leucine at position two, the MON 94313 DMO protein has an identical sequence to the wild-type DMO protein from the DI-6 strain of *S. maltophilia* (Herman *et al.*, 2005) (**Figure 6**). It is also identical to MON 87708 DMO (fully processed isoform), except for a methionine that remained at the N-terminus from the MON 94313 DMO protein, and two single amino acid changes at positions 2 and 112 (**Figure 6**). This means that MON 94313 DMO protein has a high level of sequence identity with DMO proteins previously assessed and present in biotechnology-derived crops that were reviewed by several global regulatory agencies[13], including the EFSA[14]. The differences in the amino acid sequence among the wild-type DMO, and DMOs in MON 87708 soybean, MON 87419 maize, MON 87429 maize, MON 94100 oilseed rape and MON 94313 soybean are not anticipated to have an effect on the structure of the catalytic site, functional activity, or specificity because the N-terminus and position two are sterically distant from the catalytic site (D'Ordine *et al.*, 2009; Dumitru *et al.*, 2009; Wang *et al.*, 2016) (**Figure 6**). Functional equivalence between MON 94313 DMO and MON 87429 DMO was demonstrated (*see* Section 1.4.1.5), showing that the small differences in DMO amino acid sequence do not impact the catalytical activity of enzyme. Thus, prior safety assessments of the DMO protein expressed in other biotechnology-derived crops are directly applicable to the DMO protein expressed in MON 94313.

The demethylation of DMO is very specific to dicamba. The active form of DMO is a trimer (Chakraborty et al., 2005; Dumitru et al., 2009), including for MON 94313 DMO, to be functionally active and confer dicamba tolerance. The specificity of DMO for dicamba, as with many enzymes, is due to the specific interactions that occur at the catalytic site (D'Ordine et al., 2009; Dumitru et al., 2009). The catalytic site of DMO in MON 94313 is the same as the catalytic site of DMO in wild type because the differences on the amino acid sequence between these two forms of the protein occur at position 2 and 112, which are sterically distant from the catalytic site and consequently do not participate in substrate coordination (D'Ordine et al., 2009; Dumitru et al., 2009). Dicamba interacts with amino acids in the catalytic site of DMO through both the carboxylate moiety and the chlorine atoms of dicamba, which are primarily involved in orienting the substrate in the catalytic site. These chlorine atoms are required for catalysis (D'Ordine et al., 2009; Dumitru et al., 2009). Potential substrates (o-anisic acid, vanillic acid, syringic acid, ferulic acid and sinapic acid) based on structural similarity to dicamba which are found in plants, were not metabolized by a histidine-tagged *E. coli*-produced DMO. Lack of activity for *o*-anisic acid, which is the plant metabolite most structurally related to dicamba, was also confirmed for MON 94313 DMO, supporting the specificity of MON 94313 DMO for dicamba. Given the limited existence of chlorinated compounds with structures similar to dicamba in plants and other eukaryotes (Wishart, 2010; Wishart et al., 2009), it is unlikely that MON 94313 DMO catalyzes the conversion of endogenous compounds. Therefore, the activity of the enzyme is considered specific for dicamba.

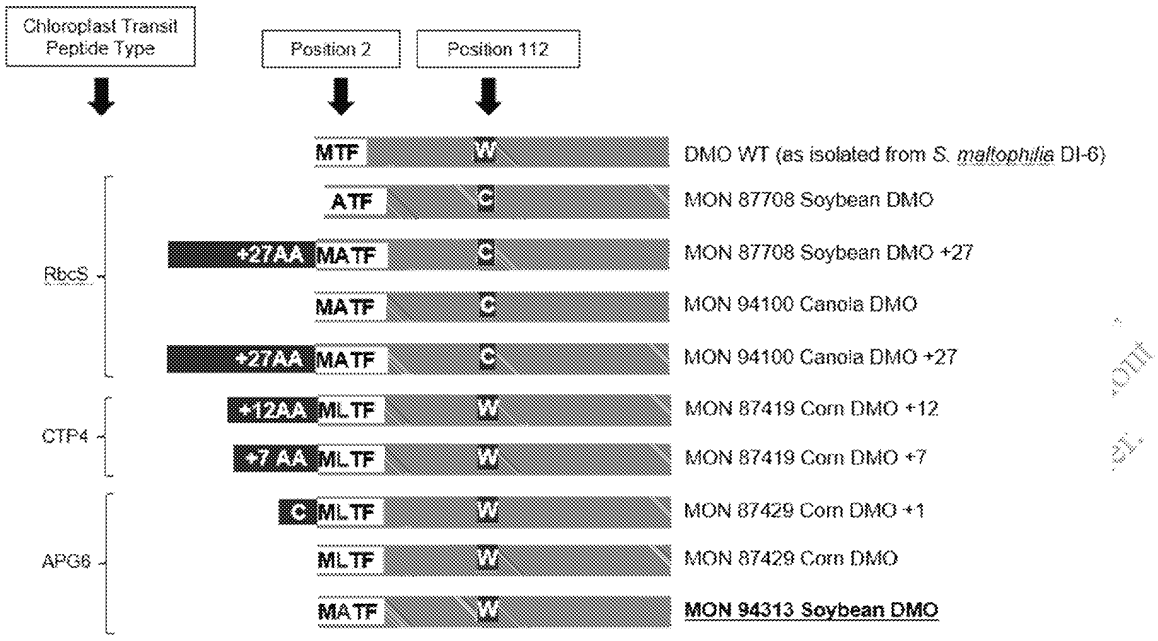


Figure 6. Forms of DMO protein and their relation to the wild-type DMO protein

Protein sequence alignment of DMO proteins in *Stenotrophomonas maltophilia,* MON 87708 soybean, MON 94100 oilseed rape, MON 87419 maize, MON 87429 maize and MON 94313 soybean. Blue indicates regions with 100% sequence identity to the wild type DMO. Insertion of a single alanine or leucine amino acid at position 2 from the N-terminus is indicated in the yellow box (A or L), and a single tryptophan to cysteine amino acid change at position 112 is indicated in red (C or W). RbcS, CTP4, and APG6 are chloroplast transit peptide sequences.

PAT protein

MON 94313 contains a *pat* gene from *S. viridochromogenes* that expresses PAT protein (**Figure 3**) to confer tolerance to glufosinate-based herbicides. The PAT protein is an acetyltransferase that catalyses the acetylation of the herbicide glufosinate to produce non-herbicidal N-acetyl glufosinate (Thompson *et al.*, 1987; Wehrmann *et al.*, 1996). PAT proteins have been isolated from two separate species of *Streptomyces*, *S. hygroscopicus* (Thompson *et al.*, 1987) and *S. viridochromogenes* (Wohlleben *et al.*, 1988). The PAT protein isolated from *S. hygroscopicus* is encoded by the *bar* gene, and the PAT protein isolated from *S. viridochromogenes* is encoded by the *pat* gene. These PAT proteins are made up of 183 amino acids with 85% identity at the amino acid level (Wohlleben *et al.*, 1988). Based on previous studies (Wehrmann *et al.*, 1996) that have extensively characterised PAT proteins produced from *bar* and *pat* genes, OECD recognises both proteins to be equivalent with regard to function and safety[22]. The PAT protein produced in MON 94313 is encoded by the *pat* gene and is identical to the wild type PAT protein encoded by *S. viridochromogenes* except for the first methionine, which is removed due to co-translational processing in MON 94313, which results in a single polypeptide of 182 amino acids that has an apparent molecular weight of

[22] OECD, 1999. Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. ENV/JM/MONO(99)13, Organisation for Economic Co-operation and Development, Paris, France. 1-26 – Accessed on 04 August 2022.

~22.4 kDa. N-terminal methionine cleavage is common and naturally occurs in the vast majority of proteins (Meinnel and Giglione, 2008). The PAT protein in MON 94313 is also produced in several commercially available glufosinate tolerant products[13,14] and therefore has an extensive history of safe use (Hérouet *et al.*, 2005; ILSI-CERA, 2011)[20]. Thus, these prior safety assessments for the PAT protein are directly applicable to the PAT protein expressed in MON 94313.

The mode-of-action for PAT protein has been extensively assessed, as numerous glufosinate-tolerant products have been reviewed by several global regulatory agencies[13] including the EFSA[14]. The PAT proteins are highly specific for glufosinate in the presence of acetyl CoA (Thompson *et al.*, 1987; Wehrmann *et al.*, 1996). Glufosinate is a racemic mixture of the D- and L-forms of phosphinothricin. The herbicidal activity of glufosinate results from the binding of L-phosphinothricin to glutamine synthetase[22, 23]. Expression of the PAT protein results in the ability to convert L-phosphinothricin to the non-herbicidal N-acetyl-L-phosphinothricin, thus conferring glufosinate tolerance to the crop. Other L-amino acids are unable to be acetylated by PAT protein and competition assays containing glufosinate, high concentrations of other amino acids and PAT showed no inhibition of glufosinate acetylation (Wehrmann *et al.*, 1996). Furthermore, the presence of L-glutamate, an analogue of glufosinate, also showed no inhibition of glufosinate acetylation in competition assays (Wehrmann *et al.*, 1996). Recent metabolic profiling reported some non-specific PAT mediated acetylation of two amino acids (aminoadipate and tryptophan) in senescent leaf extracts from *A. thaliana* and also in PAT (pat)-expressing soybean (Christ *et al.*, 2017). However, the activity level for these two amino acids was very low relative to the activity for glufosinate, indicating that PAT has a very high level of specificity for the herbicidal molecule.

FT_T.1 protein

MON 94313 contains a *ft_t.1* gene that expresses the FT_T.1 protein (**Figure 4**) to confer tolerance to 2,4-D herbicide. Similar to the RdpA protein, the FT_T.1 protein catalyses the dioxygenase reaction that degrades 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin herbicide, into herbicidally-inactive 2,4-dichlorophenol (2,4-DCP) and glyoxylic acid in the presence of alpha-ketoglutarate and oxygen (**Figure 7**). As shown in **Figure 7**, the FT_T protein also catalyses a dioxygenase reaction in the presence of αKG and oxygen to metabolize quizalofop, a FOP herbicide, into the herbicidally-inactive quizalofop phenol and pyruvate. Succinate and carbon dioxide are released as products of this reaction.

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[23] OECD, 2002. Module II: Herbicide biochemistry, herbicide metabolism and the residues in glufosinate-ammonium (Phosphinothricin)-tolentant transgenic plants. ENV/JM/MONO(2002)14, Organisation for Economic Co-operation and Development, Paris, France. – Accessed on 04 August 2022.

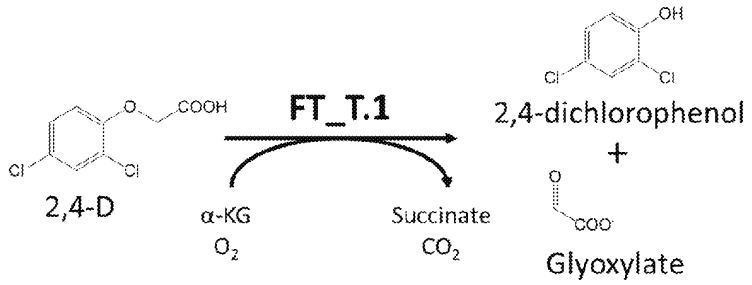


Figure 7. Substrate and metabolites of FT_T.1 protein reaction with 2,4-D

FT_T.1 in MON 94313 soybean is based off the sequence of the FT_T protein in MON 87429 maize. Both FT_T and FT_T.1 proteins share a common high order structure of Fe(II)/alpha-ketoglutarate-dependent dioxygenases that contains a classical dioxygenase active pocket including an iron atom coordinated by two histidine residues and one aspartic acid or glutamate residue (Hausinger, 2004). These Fe(II)/alpha-ketoglutarate-dependent dioxygenases have been identified in a broad range of organisms including bacteria, fungi, plants, and vertebrates, which have been extensively consumed by both humans and animals (Hausinger, 2004; Kundu, 2012) without any reports of adverse effects without any reports of adverse effects. The FT_T protein is a modified version of RdpA from the common soil bacterium *Sphingobium herbicidovorans*. FT_T was created through modifications to the RdpA amino acid sequence to improve the enzyme activity towards herbicide substrates, including 2,4-D, and to improve the stability of the enzyme at temperatures during the hotter summer growing months (Larue *et al.*, 2019). In contrast to conventional maize, which displays some natural tolerance to 2,4-D herbicide, dicots, such as soybeans, are highly sensitive to 2,4-D. Thus, FT_T was further modified to enhance its enzymatic activity to 2,4-D herbicide. The result was FT_T.1, which provides the 2,4-D tolerance in MON 94313 soybean necessary for in-crop herbicide applications (Larue *et al.*, 2019); FT_T.1 in this publication is referred to as FT_Tv7).

The FT_T.1 protein in MON 94313 soybean differs from FT_T protein in MON 87429 maize by 3 amino acids (**Figure 8**), resulting in an increase in the relative enzymatic activity of the FT_T.1 protein for 2,4-D relative to FT_T. These minor amino differences between the FT_T.1 and FT_T proteins expressed in MON 94313 and MON 87429, are not anticipated to have an effect on the structure, immunoreactivity or specificity of the protein (D'Ordine *et al.*, 2009; Dumitru *et al.*, 2009).

FT_T.1 protein expressed in MON 87429 maize, has been assessed by several global regulatory agencies around the world[13]. These agencies determined that the food and feed products derived from MON 87429 maize and its progeny are as safe and nutritious as food and feed derived from conventional maize. MON 87429 maize is under assessment by the EFSA[14].

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Figure 8. FT_T.1 relation to the FT_T protein

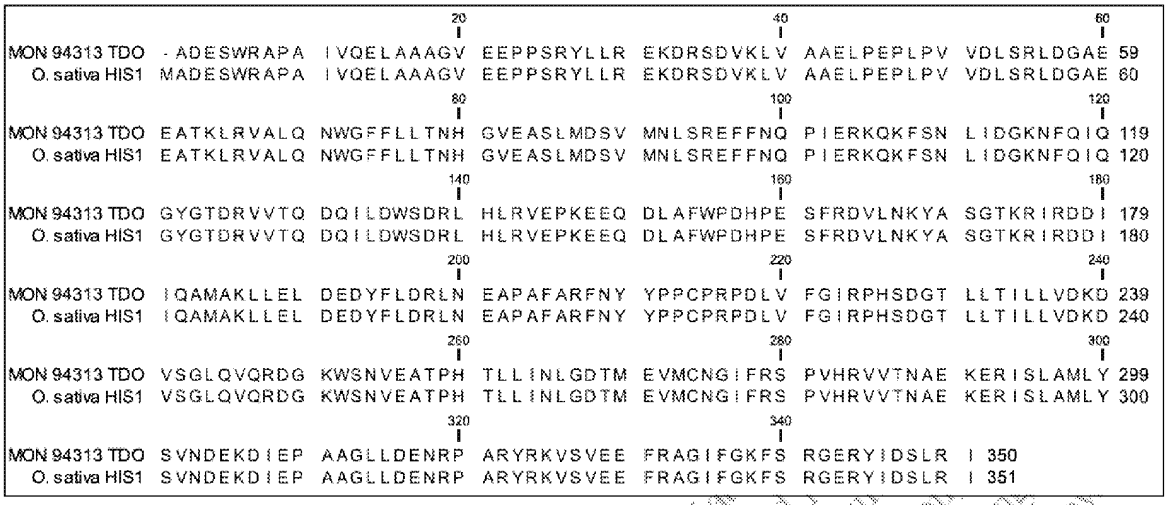
Protein sequence alignment of the FT_T protein expressed in MON 87429 maize and FT_T.1 protein expressed in MON 94313 soybean. Blue indicates regions with 100% sequence identity in amino acid sequence. Changes in specific amino acid at positions Y105F, A112T and Q246K, respectively, are indicated in red. The additional alanine (A) at the N-terminus of the FT_T protein is a result of incomplete processing of the Mdh chloroplast transit peptide.

TDO protein

The *TDO* gene provides tolerance to mesotrione. Mesotrione, a β-triketone herbicide, is an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD; EC: 1.13.11.27) which catalyses the first committed step of tyrosine catabolism. Inhibition of HPPD in plants leads to a depletion of key downstream metabolites, most notably tocopherols and plastoquinone. Plastoquinone is critical for the functioning of photosystem II and carotenoid biosynthesis (Mitchell *et al.*, 2001). Carotenoids and tocopherols are, in turn, key molecules in protecting the photosynthetic machinery from oxidative damage. As a result of the loss of these protective molecules after application of mesotrione to susceptible plants, characteristic bleaching occurs, ultimately leading to plant death. Natural tolerance to β-triketone herbicides in certain *Oryza sativa* (rice) cultivars was determined to be a result of the presence of HIS1, a protein that includes motifs conserved in Fe (II)/alpha-ketoglutarate-dependent dioxygenases, through oxidation of the herbicide molecule (Maeda H, 2019).

Ectopic expression of HIS1, called TDO in MON 94313 soybean (**Figure 9**), results in soybean plants that are capable of tolerating in-crop applications of mesotrione through a sequential double oxidation of the mesotrione molecule.

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```
                                    20                  40                  60
MON 94313 TDO  -ADESWRAPA IVQELAAAGV EEPPSRYLLR EKDRSDVKLV AAELPEPLPV VDLSRLDGAE 59
O. sativa HIS1 MADESWRAPA IVQELAAAGV EEPPSRYLLR EKDRSDVKLV AAELPEPLPV VDLSRLDGAE 60
                                    80                  100                 120
MON 94313 TDO  EATKLRVALQ NWGFFLLTNH GVEASLMDSV MNLSREFFNQ PIERKQKFSN LIDGKNFQIQ 119
O. sativa HIS1 EATKLRVALQ NWGFFLLTNH GVEASLMDSV MNLSREFFNQ PIERKQKFSN LIDGKNFQIQ 120
                                    140                 160                 180
MON 94313 TDO  GYGTDRVVTQ DQILDWSDRL HLRVEPKEEQ DLAFWPDHPE SFRDVLNKYA SGTKRIRDDI 179
O. sativa HIS1 GYGTDRVVTQ DQILDWSDRL HLRVEPKEEQ DLAFWPDHPE SFRDVLNKYA SGTKRIRDDI 180
                                    200                 220                 240
MON 94313 TDO  IQAMAKLLEL DEDYFLDRLN EAPAFARFNY YPPCPRPDLV FGIRPHSDGT LLTILLVDKD 239
O. sativa HIS1 IQAMAKLLEL DEDYFLDRLN EAPAFARFNY YPPCPRPDLV FGIRPHSDGT LLTILLVDKD 240
                                    260                 280                 300
MON 94313 TDO  VSGLQVQRDG KWSNVEATPH TLLINLGDTM EVMCNGIFRS PVHRVVTNAE KERISLAMLY 299
O. sativa HIS1 VSGLQVQRDG KWSNVEATPH TLLINLGDTM EVMCNGIFRS PVHRVVTNAE KERISLAMLY 300
                                    320                 340
MON 94313 TDO  SVNDEKDIEP AAGLLDENRP ARYRKVSVEE FRAGIFGKFS RGERYIDSLR I 350
O. sativa HIS1 SVNDEKDIEP AAGLLDENRP ARYRKVSVEE FRAGIFGKFS RGERYIDSLR I 351
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Figure 9. Alignment of MON 94313 TDO and *Oryza sativa* HIS1

Alignment of amino acid sequences of HIS1 protein (Os02g0280700) from rice (https://www.uniprot.org/uniprot/A3A5K1) and the TDO protein from MON 94313 soybean. TDO and rice HIS1 are identical at the amino acid level, except for the N-terminal M at position #1 which is cleaved in MON 94313 soybean TDO during protein processing in the cytosol.

Like other Fe (II)/alpha-ketoglutarate-dependent dioxygenases, TDO uses iron and alpha-ketoglutrate as cofactors in the oxidation of its substrate, producing succinate and the oxidized product. Alpha-ketoglutarate-dependent non-heme iron dioxygenases share a common double-stranded, beta-helix protein fold with three metal-binding ligands found in a His1-X-Asp/Glu-Xn-His2 motif. In oxygenation reactions, alpha-ketoglutarate (αKG) chelates Fe(II) using its C-1 carboxylate and C2-ketone. Decarboxylation of αKG results in the formation of succinate and carbon dioxide, which leads to the generation of an Fe(IV)-oxo or other activated oxygen species that subsequently hydroxylate the primary substrate, *e.g.*, mesotrione (Bugg, 2003; de Carolis and de Luca, 1994). *In vitro* studies of recombinant TDO show that the initial oxidation of mesotrione takes place at the 5'C, yielding 5' hydroxy-mesotrione (Maeda H, 2019). A second oxidation, followed by spontaneous loss of a water molecule, creates oxy-mesotrione (*see* **Figure 10**). *In vitro* assays demonstrated that hydroxy-mesotrione is less inhibitory to HPPD than mesotrione, and oxy-mesotrione had virtually no impact on the activity of HPPD. Thus, the oxidative capabilities of TDO provide a mechanism by which mesotrione is neutralized and prevented from inhibiting the native soybean HPPD activity.

Studies of soybean expressing the TDO protein show that the plants can tolerate in-crop applications of mesotrione (Dai *et al.*, 2022). Metabolic analysis of soybean leaves expressing TDO demonstrated that tolerance is accomplished through rapid conversion of mesotrione primarily to hydroxy-mesotrione, hydroxy-xanthone, and downstream glucosyl and malonyl conjugates (oxy-mesotrione is not detected in TDO-expressing soybean, likely due to non-enzymatic cyclization to hydroxy-xanthone). In addition, mesotrione is not translocated from the treated tissues in TDO-expressing soybean, thus preventing inhibition of HPPD in newly developing apex or root tissues. In contrast, apical and root tissues of treated conventional soybean accumulate mesotrione, resulting in the inhibition of HPPD and the bleaching phenotype typical of β-triketone treatments of susceptible plants (Dai *et al.*, 2022).

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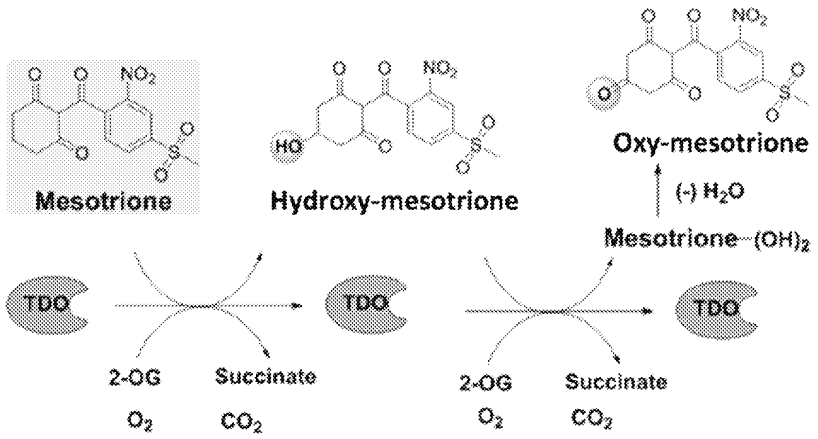


Figure 10. MON 94313 TDO biochemical mode of action

TDO oxidizes mesotrione sequentially, yielding hydroxy-mesotrione in the first round of oxidation, followed by oxy-mesotrione after the second oxidative step and the loss of a water molecule. Hydroxy- and oxy-mesotrione are progressively less inhibitory to HPPD than the parent mesotrione molecule, providing MON 94313 soybean with tolerance to in-crop application of mesotrione. Oxy-mesotrione is not detected in treated leaves, likely due to non-enzymatic conversion to hydroxy-xanthone, which is further metabolized to glucosyl and malonyl conjugates (Dai *et al.*, 2022) (2-OG = alpha-ketoglutarate).

To examine the substrate specificity of the TDO protein, a combined *in silico* and *in vitro* assessment was conducted to identify potential endogenous substrates. This consisted of a 2-dimensional (2D) and 3-dimensation (3D) screen of the NAPRALERT® plant metabolite database, which includes soybean metabolites, for molecules that met 2D criteria set by a known TDO substrate (mesotrione) and expectations of the 3D mesotrione conformation in the TDO active site. A schematic of the *in silico* screening approach is shown in **Figure 11**. **Table 1** lists the 59 plant metabolites from the database with the highest combined 2D and 3D scores, and therefore the highest probability of being a substrate for TDO. Commercially available candidate compounds identified in the plant metabolite database (a total of 32 molecules), the positive control (mesotrione) and other potential HPPD herbicide substrates were tested *in vitro* to assess the specificity of the TDO protein. The results showed that TDO had no significant activity with any of the putative plant substrates, but was specific to the β-triketone class of HPPD herbicide compounds (*e.g.,* mesotrione, tembotrione, and sulcotrione) (*see* **Table 2**).

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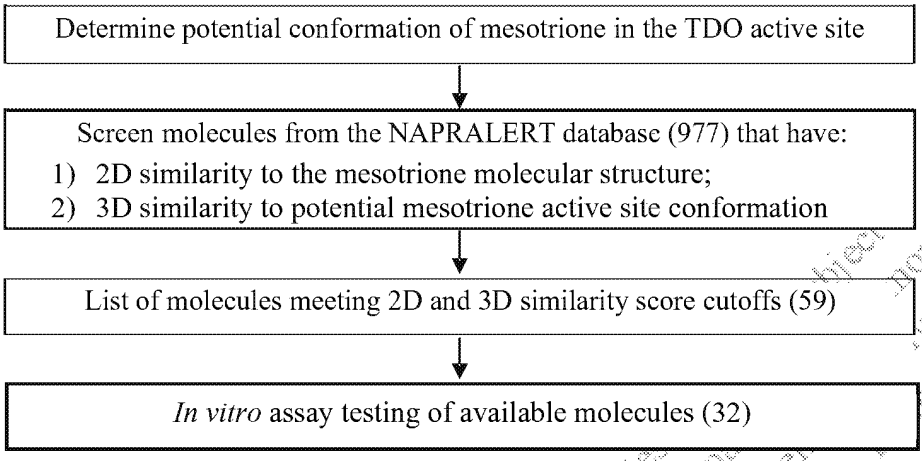


Figure 11. *In silico* and *in vitro* protocol for TDO endogenous substrate specificity screen

Overview of the screening and testing scheme to identify putative endogenous plant small molecules that could act as substrates for the TDO enzyme.

Table 1. *In silico* plant metabolites identified as potential TDO substrates based on 2D and 3D similarity to mesotrione chemical structure and potential active site conformation

PubChem CID	NAPRALERT® Common Name	PubChem Common Name / IUPAC Name
100985262	Cyclohexadienylketone, ethyl-iso-propyl	(1S)-1beta-ethyl-2-methoxy-2,5-cyclohexadienyl
0660412	Sinalbin	Sinalbin A
108125	Lacinilene C 7-methyl ether	Lacinilene C 7-methyl ether
115300	Hemigossypol	Hemigossypol
13067	Indole-3-acetic acid methyl ester	1H-Indole-3-acetic acid, ethyl ester
13314	Lormetazepam	Lormetazepam
15520697	Raimondalone	Raimondalone
170551	Lacinilene	Lacinilene C
176907	Closely related to daidzein	Dihydrodaidzein
17925	Delorazepam	Delorazepam
182249	Para-hemigossypolone	Para-hemigossypolone
186907	Aflatoxin	Aflatoxin B
19763563	Acetaldehyde,2-thiophene	Benzo[b]thiophene-2-acetaldehyde
2358	Benzoxazin-3(4H)-one,1-4:(2H): 2-4-dihydroxy-7-methoxy	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one
25200808	Indole-3-acetyl-glutamate	Indole-3-acetyl-glutamate(2-)
25201975	Closely related to tryptophan,d:N-malonyl	N-(carboxylatoacetyl)-D-tryptophanate
25202708	Heliocide H2	Heliocide H2

PubChem CID	NAPRALERT® Common Name	PubChem Common Name / IUPAC Name
25202994	Heliocide H3	Heliocide H3
25203649	Hemigossypolone	Hemigossypolone-6-methyl ether
2997	Diazepam, demethyl	Demethyldiazepam
3080590	Oxindole-3-acetic acid	Oxindole-3-acetic acid
441140	Griseofulvin	Griseofulvin
44257530	Phaseol	Phaseol
46173376	Agmatine, feruloyl	Feruloylagmatine
493570	Riboflavin	Riboflavin
4947	Gallate,N-propyl	Propyl gallate
520156	Closely related to naphthone,2-aceto-1-methyl	2,6-Dimethyl-1-aceto-naphthone
5280373	Biochanin A	Biochanin A
5280378	Formononetin	Formononetin
5280385	Sinapine	Sinapine
5280460	Scopoletin	Scopoletin
5280961	Genistein	Genistein
5281254	Hispidol	Hispidol
5281576	Zearalenone	Zearalenone
5281704	Afromosin	7-hydroxy-6-methoxy-3-(4-methoxyphenyl)-4H-chromen-4-one
5281707	Coumestrol	Coumestrol
5281708	Daidzein	Daidzein
5281809	Sojagol	Sojagol
5317750	Glycitein	Glycitein
5367698	Closely related to hex-cis-3-en-1-ol acetate	cis-3-hexen-1-yl phenylacetate
5458878	Tryptamine,N-(para-coumaroyl)	p-coumaroyltryptamine
5870	Estrone	Estrone
613611	Diazepam,iso	Iso-diazepam
62428	Closely related to naphthalene,1-ethyl	Ethyl 1-naphthaleneacetate
633898	Closely related to luteolin	Trimethyl-.beta.-isoluteone
6436348	Germacrone	Germacrone
6452650	Diazepam	Diazepam hydrochloride
6455001	Closely related to apigenin	Anthracenecarboxaldehyde
6781	Phthalate, ortho diethyl	Diethyl phthalate
6985	Acetophenone, 2-4-dimethyl	2,4-Dimethylacetophenone
72187670	Closely related to apigenin	(5S,8aR,10aS)-2,3-Dihydroxy-4-isopropyl-6,10a-dimethyl-5-(3-methyl-2-butenyl)-9,10-dioxo-5,8,8a,9,10,10a-hexahydro-1-anthracenecarbaldehyde
730037	Indole-3-butyric acid	Indole-3-(4'-oxo)butyric acid

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PubChem CID	NAPRALERT® Common Name	PubChem Common Name / IUPAC Name
74706	Indole-3-acetic acid	Indole-3-acetic acid
8441	Terephthalate, dimethyl:	Dimethyl terephthalate
8554	Phthalate, dimethyl	Dimethyl phthalate
86223063	Chitinase-IN-1	Chitinase-IN-1
86223064	Chitinase-IN-2	Chitinase-IN-2
896	Melatonin	Melatonin
932	Naringenin	Naringenin

Table 2. Results of *in vitro* TDO assays with available plant metabolites identified in the *in silico* screen.

PubChem ID	PubChem Common Name/ IUPAC Name	Mean	Estimate	StdErr	p-value	Sig
176907	Dihydrodaidzein	-0.000064333	0.2974	0.5124	0.577	
5281254	Hispidol	-0.000017889	-1.1079	0.5124	0.0612	
86223063	Chitinase-IN-1	-0.000039778	-0.07912	0.1096	0.48	
25200808	Indole-3-acetyl-glutamate(2-)	-0.000034556	-0.211	0.1096	0.0708	
6436348	Germacrone	-0.000042889	0.004319	0.1096	0.969	
5317750	Glycitein	-0.000114889	0.5093	0.5124	0.3483	
5280373	Biochanin A	-0.000045167	0.08054	0.1245	0.5262	
6781	Diethyl phthalate	-0.000041222	-0.03704	0.1096	0.7395	
74706	Indole-3-acetic acid	-0.000036	-0.1721	0.1096	0.1345	
84098	Isoxaflutole	-0.000062889	0.1877	0.5124	0.7232	
62428	Ethyl 1-naphthaleneacetate	-0.000042444	-0.0066	0.1096	0.9527	
	DMSO	-0.000043111				
	Mesotrione	-0.000158889	1.2798	0.1096	<.0001	*
3080590	Oxindole-3-acetic acid	-0.000026	0.1013	0.2944	0.7337	
4947	Propyl gallate	-0.000028444	0.2025	0.2944	0.4978	
5281576	Zearalenone	-0.000035778	0.2464	0.2944	0.4106	
896	Melatonin	-0.000019111	-0.1913	0.2944	0.5218	
8554	Dimethyl phthalate	-0.000023667	-0.04499	0.2944	0.8798	
5870	Estrone	-0.000027567	0.173	0.2944	0.562	
5281708	Daidzein	-0.000042222	0.5873	0.2944	0.0571	
5281707	Coumestrol	-0.000023167	-0.0012	0.3324	0.9972	
5280460	Scopoletin	-0.000023333	0.02116	0.2944	0.9433	
5280961	Genistein	-0.000028667	0.2146	0.2944	0.4729	
13067	1H-Indole-3-acetic acid, ethyl ester	-0.000021111	-0.08247	0.2944	0.7817	
	DMSO	-0.000023778				
	Mesotrione	-0.000127444	1.7007	0.2944	<.0001	*

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PubChem ID	PubChem Common Name/ IUPAC Name	Mean	Estimate	StdErr	p-value	Sig
92383	Pyrazoxyfen	-0.000044667	0.1917	0.1298	0.1509	
11556911	Tembotrione	-0.000131111	1.2794	0.1298	<.0001	*
91760	Sulcotrione	-0.000124444	1.2287	0.1298	<.0001	*
932	Naringenin	-0.000033667	-0.08098	0.1298	0.5377	
6985	2',4'-Dimethylacetophenone	-0.000036556	0.006742	0.1298	0.9589	
441140	Griseofulvin	-0.000040333	0.1025	0.1298	0.4365	
730037	Indole-3-(4'-oxo)butyric acid	-0.000040333	0.09884	0.1298	0.4527	
170551	Lacinilene C	-0.000032778	-0.1043	0.1298	0.4282	
5280385	Sinapine	-0.000036	-0.01244	0.1298	0.9243	
11302979	Topramezone	-0.000031111	-0.1869	0.1298	0.1609	
	DMSO	-0.000036333				
	Mesotrione	-0.000147778	1.4006	0.1298	<.0001	*
493570	Riboflavin	-8.74997E-05	-0.03105	0.03589	0.8039	
5280378	Formononetin	-0.000085395	-0.04679	0.03589	0.9001	
8441	Dimethyl terephthalate	-8.73703E-05	-0.02213	0.03589	0.7295	
5281704	7-hydroxy-6-methoxy-3-(4-methoxyphenyl)-4H-chromen-4-one	-0.000083938	-0.0672	0.03589	0.9657	
86223064	Chitinase-IN-2	-8.61973E-05	-0.04682	0.03589	0.9002	
	DMSO	-8.98513E-05				
	Mesotrione	-0.000394938	1.3657	0.08844	<.0001	*

Enzymatic activity assays were conducted for each potential plant metabolite and six HPPD inhibitor herbicidal molecules (including mesotrione). The table represents four different sets of data, each containing a mesotrione positive control (shaded in green) and a dimethyl sulfoxide (DMS) negative control. Other HPPD inhibitor herbicidal molecules are shaded in orange. Statistical comparisons were done between the estimate (log transformed change in absorbance) for each molecule and the negative control for each assay. Statistical significance was based on a p value score <0.05.

1.2.2.2. *Information on the sequences actually inserted/deleted*

Characterisation of the DNA insert in MON 94313 was conducted using a combination of sequencing, polymerase chain reaction (PCR), and bioinformatics (M-815764-02-1, 2022).

Information related to the quality of DNA sequencing following the instructions provided in EFSA's technical note[24] is provided in "DNA Sequencing_info.7z" file.

The results of this characterisation demonstrate that MON 94313 contains one copy of the T-DNA I containing the *dmo, pat, ft_t.1* and *TDO* expression cassettes that is stably inherited over multiple generations and segregates according to Mendelian principles.

[24] EFSA, 2018. Technical Note on the quality of DNA sequencing for the molecular characterisation of genetically modified plants. The EFSA Journal, 16(7):5345, 1-11. - Accessed on 04 August 2022.

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Segregation analysis of the T-DNA was conducted to determine the inheritance and generational stability of the insert in MON 94313. Segregation analysis corroborates the insert stability demonstrated by Next Generation Sequencing (NGS) and independently establishes the genetic behaviour of the T-DNA (M-816874-02-1, 2022).

a) Size and copy number of all detectable inserts, both complete and partial

The number of inserted DNA sequences from PV-GMHT529103 in MON 94313 was assessed by generating a comprehensive collection of reads via NGS of MON 94313 genomic DNA using the R3 generation (M-815764-02-1, 2022). This study was conducted in compliance with OECD principles on Good Laboratory Practice (GLP) or by an International Organisation for Standardisation (ISO) accredited laboratory. A plasmid map of PV-GMHT529103, a schematic representation of the insert and flanking sequences in MON 94313 and descriptions of the genetic elements present in MON 94313 are provided in M-815764-02-1 (2022). The generations studied are depicted in the breeding history diagram shown in Section 1.3.1.

b) Organisation and sequence of the inserted genetic material at each insertion site

The organisation and sequence of the elements within the DNA insert and the adjacent genomic DNA was assessed using directed DNA sequence analysis (M-815764-02-1, 2022). The results of this analysis confirm that the MON 94313 insert is 10 196 bp and that each genetic element within the T-DNA is intact compared to PV-GMHT529103, with the exception of the border regions. The border regions both contain small terminal deletions with the remainder of the inserted border regions being identical to the sequence in PV-GMHT529103.

c) In the case of deletion(s), size and function of the deleted region(s), whenever possible

A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5′ and 3′ flanking sequences of MON 94313 indicates that 40 bases of soybean genomic DNA were deleted during integration of the T-DNA (M-815764-02-1, 2022).

d) Subcellular location(s) of insert(s) (nucleus, chloroplasts, mitochondria, or maintained in a non-integrated form) and methods for its/their determination

The presence of MON 94313 insert in the nuclear genome is best shown by the Chi square (χ^2) analysis of the segregation results (*see* Section 1.2.2.4). The χ^2 analysis of the segregation pattern, according to Mendelian genetics, was consistent with a single site of insertion into soybean nuclear DNA.

e) Sequence information for both 5' and 3' flanking regions at each insertion site, with the aim of identifying interruptions of known genes

Sequence information

The MON 94313 insert sequence characterising the integrated DNA in MON 94313 is presented in M-815764-02-1 (2022).

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Bioinformatic analysis of MON 94313 sequence flanking the insert

The genomic DNA sequence flanking the insertion site of the MON 94313 was evaluated using FASTA analyses to determine if the insertion of the T-DNA had disrupted any endogenous ORFs located in the soybean genomic DNA flanking the insert, or whether ORFs from the soybean genome are present in the flanking genomic DNA adjacent to the T-DNA after transformation (TRR0001383, 2022).

The FASTA analyses were conducted using the GMA_2021 database with the aim of performing both intraspecies and interspecies similarity searches (TRR0000689, 2021). The results indicated that no endogenous elements were disrupted and the MON 94313 T-DNA is placed within chromosome 13 of the soybean genome.

f) Open Reading Frames (ORFs) created as a result of the genetic modification either at the junction sites with genomic DNA or due to internal rearrangements of the insert(s)

Bioinformatics assessment of the T-DNA insert

The T-DNA sequence was translated on six reading frames. Each translated sequence was used as a query for a FASTA, and an eight- and an 80-amino acid sliding window searches against the AD_2021 database and a FASTA search against the TOX_2021 and PRT_2021 databases. A complete description of the FASTA comparisons and the sliding window searches can be found in TRR0001382 (2022).

The results of these data indicate that no biologically relevant sequence similarities were observed between the six reading frames translated from the T-DNA and allergens, toxins, or biologically active proteins associated with adverse effects for human or animal health.

Other than translation of MON 94313 DMO, FT_T.1, TDO and PAT no evidence exists to indicate that any other sequence from the T-DNA is translated. Rather, the results of these bioinformatic analyses indicate that in the unlikely occurrence that any of the sequences analysed herein is found *in planta*, or translation of sequence other than the intended products was to occur, none would share significant similarity or identity to known allergens, toxins, or other biologically active proteins that could affect human or animal health .

Bioinformatics analysis of the genomic DNA – insert junctions

As part of a comprehensive safety assessment, bioinformatic analyses were performed to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by the 5′ and 3′ inserted DNA-soybean genomic DNA junctions. Sequences spanning the 5′ and the 3′ soybean genomic DNA/T-DNA junctions were translated from stop codon to stop codon in all six reading frames. The putative polypeptides were compared to AD_2021, TOX_2021 and PRT_2021 databases using the FASTA algorithm and to the AD_2021 database using an eight- and an 80-amino acid sliding window searches. A complete description of the FASTA comparisons and the sliding window searches can be found in TRR0001381 (2022).

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The results of these data indicate that no biologically relevant sequence similarities were observed between the translated putative flank junction sequences and allergens, toxins, or biologically active proteins associated with adverse effects for human or animal health. The bioinformatic analyses performed using the putative sequences translated from junctions is theoretical. Likewise, other than translation of MON 94313 DMO, FT_T.1, TDO, and PAT no evidence exists to indicate that any other sequence from the T-DNA is translated. Rather, the results of these bioinformatic analyses indicate that in the unlikely occurrence that any of the putative flank junction sequences analysed herein is found in planta, or translation of sequence other than the intended protein products was to occur, none would share significant similarity or identity to known allergens, toxins, or other biologically active proteins that could affect human or animal health.

An overview table summarising the algorithms and databases used in the bioinformatics analyses performed with the MON 94313 is included in **Appendix 3**.

1.2.2.3. *Information on the expression of the insert(s)*

For analysis of the expression of the insert, MON 94313 tissue samples, treated and not treated with intended herbicides, were collected from five 2020 US field trials (TRR0001361, 2022). Those sites were representative of soybean producing regions suitable for commercial production. Site information is decribed in the phenotypic evaluation and environmental interactions of soybean MON 94313 in 2020 U.S. field trials report (*see* Section 1.3.2). Details on the newly expressed protein levels in forage and grain as well as in flower can be found in TRR0001507 (2022a). The study was conducted in compliance with OECD principles of GLP.

a) The methods used for expression analysis together with their performance characteristics

MON 94313 DMO, PAT, FT_T.1 and TDO protein expression levels were determined by validated multiplexed immunoassay (MON 94313 DMO, PAT and FT_T.1) or enzyme linked immunosorbent assay (ELISA) (TDO) in tissues, treated and not treated with intended herbicides, collected from MON 94313 produced in 2020 US field trials (TRR0001507, 2022a).

The immunoassay methods utilised were developed and validated to ensure that they were specific for the protein of interest. Each individual analysis is executed utilising the corresponding protein standards for quantification as well as negative and positive assay controls that ensure the assay is functioning within the validated specifications.

The methods were optimised to minimise method bias. Protein extracts from the test substances were analysed by immunoassay with the appropriate protein standard and inter-assay negative and positive controls. Various assays that are performed during development and validation of the immunoassays assure that accurate protein of interest (POI) expression levels are determined for each tissue type tested. Various experiments are performed to ensure that the immunoassay is specific for the POI and that there are no matrix effects that could lead to inaccuracies in the expression values reported. Antibody specificity for the POI is determined during the development and validation of the immunoassays to be used in expression studies. During development many antibodies are screened to ensure

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that they are specific for the POI and not cross-reactive with proteins in the conventional matrix. The antibodies selected are used during the final validation of the immunoassays in a series of experiments that utilise the conventional counterpart, to confirm that these antibodies do not cross-react with proteins in the conventional counterpart. The experiments are performed with the tissue type that is analysed during the expression study. They must meet the validation criteria or the method validation would fail and additional method development would be required. Any concerns regarding non-specific binding of the antibody to proteins in conventional tissues have been addressed by the validation experiments performed for each immunoassay.

Once validated, the multiplexed immunoassay (MON 94313 DMO, PAT and FT_T.1) or ELISA (TDO) are utilised in the expression study. Each immunoassay performed contains assay controls, a standard curve, and the samples to be assayed. The assays controls consist of a negative control (QC-) and a positive control (QC+). The QC- is an extract prepared from tissue that does not contain the POI, where the values obtained for the QC- sample must fall below the limit of quantification (<LOQ), or the plate is rejected. The QC+ sample is a transgenic plant tissue extract that generates a known protein concentration range. Together these controls ensure that the assay is not affected by the matrix (QC-) and that it is working properly, within the parameters determined during validation (QC+); confirming the data obtained during validation every time the samples are analysed.

For more detailed information on the performance characteristics of the assays, please refer to TRR0001507 (2022a).

b) Information on developmental expression of the insert during the life cycle of the plant

The plant parts selected for protein expression include forage and grain collected at the BBCH 77 and BBCH 99 growth stages, respectively. These time points for collection of forage and grain are representative for these types of tissues that are the primary source of MON 94313 that will be used as food and feed. In addition, flower, used as surrogate tissue for pollen, was collected at the BBCH 61-71 growth stage.

c) Parts of the plant where the insert/modified sequences are expressed

As described in Section 1.2.2.3.(b), the expression levels of the MON 94313 DMO, PAT, FT_T.1 and TDO proteins in MON 94313 were measured in forage and grain, which are the most relevant tissues for the food and feed safety risk assessment. In addition, the expression levels in flower, used as surrogate tissue for pollen, needed for the human dietary exposure assessment for soybean pollen supplements, were measured. Finally, expression levels were assessed in other tissue types that are not relevant for the scope of this application (TRR0001361, 2022).

d) Potential unintended expression of new ORFs identified under point 1.2.2.2.(f) which raise a safety concern.

Not applicable, as described in Sections 1.2.2.2.(e)and 1.2.2.2.(f), no newly expressed ORFs that could raise any safety concern for human or animal health or the environment were identified.

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e) Protein expression data, including the raw data, obtained from field trials and related to the conditions in which the crop is grown.

The protein expression levels in the relevant tissues for the risk assessment are presented in TRR0001507 (2022a). Raw data for the study can be found in TRR0001507 (2022b). The breeding diagram of MON 94313 showing the generations used for this study is provided in Section 1.3.1.

A summary table with information regarding the field trial related to the newly expressed protein levels is provided in **Appendix 4**.

Taken together, these facts demonstrate that the information presented is adequate to assess the food and feed safety of MON 94313 imported into the EU in terms of protein expression.

1.2.2.4. *Genetic stability of the insert and phenotypic stability of the genetically modified plant*

a) Genetic stability of the insert

In order to demonstrate the genetic stability of the T-DNA present in MON 94313 through multiple breeding generations, NGS was performed using DNA obtained from five breeding generations of MON 94313. The generations studied are depicted in the breeding history diagram shown in Section 1.3.1. Details on the genetic stability study are described in M-815764-02-1 (2022).

These results of the genetic stability study demonstrate that the single locus of integration characterised in the R3 generation of MON 94313 is found in five breeding generations of MON 94313, confirming the stability of the insert. This comprehensive NGS and bioinformatic analysis of NGS data from multiple generations supports the conclusion that MON 94313 contains a single and stable T-DNA insert.

b) Phenotypic stability of the genetically modified plant

The MON 94313 T-DNA resides at a single locus within the soybean genome and therefore should be inherited according to Mendelian principles of inheritance. During development of lines containing MON 94313, phenotypic and genotypic segregation data were recorded to assess the inheritance and stability of the MON 94313 T-DNA using Chi square (χ^2) analysis over several generations. The χ^2 analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles. The MON 94313 breeding path for generating segregation data is described in the breeding history diagram shown in Section 1.3.1. Details on the phenotypic and genotypic segregation study are described in M-816874-02-1 (2022). This study was conducted under an ISO 9001 Quality Management System.

The results of the χ^2 analysis of the segregating progeny of MON 94313 support the conclusion that the MON 94313 T-DNA I resides at a single locus within the soybean genome and is inherited according to Mendelian principles. These results are also consistent with the molecular characterisation data indicating that MON 94313 contains a single intact copy of the T-DNA inserted at a single locus in the soybean genome (*see* Section 1.2.2.2.(a)).

1.2.2.5. *Potential risk associated with horizontal gene transfer*

It is highly unlikely for DNA sequences from plants to recombine with genomic DNA in human or animal cells due to mechanisms that result in DNA degradation and physical barriers to uptake. This conclusion is supported by:

- The existence of natural barriers that inhibit the cellular uptake of exogenous DNA. Systemic barriers include stomach acid, pancreatic nucleases, intestinal epithelium, vascular endothelium and blood/systemic nucleases, and cellular barriers that include the plasma membrane, endosomal sequestration and lysosomal degradation. Each of these barriers would serve to limit and/or eliminate the availability of exogenous DNA that might be capable of recombination with human or animal cells. As a result, the probability of any foreign DNA entering and recombining with the host DNA in humans and animals is negligible.
- A study conducted on human subjects fed on genetically modified soybean has confirmed that the transgene does not survive passage through the human gastro-intestinal tracts and no evidence was found to suggest gene transfer between GM soybean and intestinal micro-flora occurred during the feeding experiments (Netherwood *et al.*, 2004).
- Transgene DNA contained in plants does not differ in its physicochemical properties when compared to endogenous plant DNA that has been consumed throughout history by humans and herbivorous and omnivorous animals. Despite this consumption of plant DNA, there are no reports plant genomic DNA integrating into the genome of a consuming human or animal.
- A number of scientific studies have been conducted to examine the fate of transgenic DNA in animals fed genetically enhanced crops. In these studies, transgenic DNA was undetectable (Jennings *et al.*, 2003a; Jennings *et al.*, 2003b; Nemeth *et al.*, 2004) or sporadically detectable (Sharma *et al.*, 2006; Zhu *et al.*, 2004) in the animal tissues that were tested.

Additionally, horizontal gene transfer (HGT) from genetically enhanced plants to environmental micro-organisms is unlikely due to the overall complexity and probability of the process. At a minimum the following must occur for homologous recombination (HR) to be of significance:

- The recipient bacteria must be competent and able to accept exogenous DNA.
- The recipient bacteria and donor plant must share DNA that is homologous. In the instance of bioinformatic analyses described in this application, the definition of homologous is that the donating plant and recipient bacteria must share an ungapped 200 bp of DNA sequence having at least 190 identical nucleotides[25].
- The sequence in the bacterial genome cannot contain essential genes that if lost due to recombination would be lethal or otherwise compromise the fitness of the recipient bacteria.

[25] EFSA, 2015. Explanatory note on DNA sequence similarity searches in the context of the assessment of horizontal gene transfer from plants to microorganisms – Accessed on 04 August 2022.

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- Assuming recombination has occurred, the gene transferred from the plant genome must provide an advantage to the recipient bacteria in the environment over its untransformed neighbours.

Each of the aforementioned steps or outcomes is of low probability and the likelihood of all steps occurring is the product of the multiplication of all probabilities. Therefore, it is concluded that HGT is unlikely.

Nonetheless, in order to assess the potential of homologous recombination (HR) between the MON 94313 T-DNA I and environmental organisms, the MON 94313 T-DNA and flanking genomic sequence were used as the query for FASTA searches of databases containing DNA sequences of archaea genomes (ARC_2021 database), bacterial genomes (BCT_2021 database) and bacteriophage viral genomes (PHG_2021 database). Details about the bioinformatic evaluation of the potential for the T-DNA HR of MON 94313 are provided in TRR0001384 (2022).

After combining all the evidence collected from the sequence analysis, it is clear that the occurrence of HR from MON 94313 to archaea, bacteria or virus is extremely unlikely. Based on the above, it is highly unlikely for DNA sequences from plants to recombine with genomic DNA in cells of humans, animals or micro-organisms.

1.2.3. Additional information relating to the genetically modified plant required for the environmental safety aspects

1.2.3.1. *Information on how the genetically modified plant differs from the recipient plant in reproduction, dissemination, survivability or other properties*

Based on centuries of experience with conventional, domesticated soybean in the EU, there is no potential for soybean to be invasive of natural habitats or persist in the agronomic environment without human intervention. Soybean is known as a poor competitor, which outside of cultivation has no meaningful impact on the environment[4]. The inserted herbicide tolerant traits in MON 94313 are not expected to alter the reproduction, dissemination or survivability characteristics of soybean.

The agronomic, phenotypic and environmental interaction evaluations are described in more detail in Section 1.3.5.

1.2.3.2. *Any change to the ability of the genetically modified plant to transfer genetic material to other organisms*

a) Plant to bacteria gene transfer

None of the genetic elements inserted in MON 94313 has a genetic transfer function. Therefore, no changes are expected in the ability of this soybean to transfer genetic material to bacteria.

b) Plant to plant gene transfer

Based on the observation that reproductive morphology in MON 94313 is unchanged compared to conventional soybean, the out-crossing frequency to other soybean varieties or to wild relatives would be unlikely to be different for MON 94313, when compared to conventional soybean varieties. Furthermore, the scope of the current application does not include the cultivation of MON 94313 in the EU.

1.2.4. Conclusions of the molecular characterisation

Characterisation of the genetic modification in MON 94313 was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterisation demonstrate that MON 94313 contains a single copy of T-DNA I containing the *dmo, pat, ft_t.1* and *TDO* expression cassettes that is stably integrated at a single locus and is maintained over multiple generations according to Mendelian principles. These conclusions are based on the following evidences:

- Molecular characterisation of MON 94313 by NGS demonstrated that MON 94313 contained a single T-DNA insert. These whole-genome analyses provided a comprehensive assessment of MON 94313 to determine the presence and identity of sequences derived from PV-GMHT529103 and demonstrated that MON 94313 contains a single T-DNA insert, with no detectable backbone from PV-GMHT529103.
- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) performed on MON 94313 was used to determine the complete sequence of the single DNA insert from PV-GMHT529103, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirmed that the organisation and sequence of the genetic elements in the MON 94313 insert are consistent with PV-GMHT529103.
- Furthermore, the genomic organisation at the insertion site in MON 94313 was assessed by comparing the sequences flanking the T-DNA insert in MON 94313 to the sequence of the insertion site in conventional soybean. This analysis determined that 40 bases of soybean genomic DNA were deleted during integration of the T-DNA.
- Generational stability analysis by NGS demonstrated that the single PV-GMHT529103 T-DNA I insert in MON 94313 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 94313.
- Segregation data confirm that the inserted T-DNA segregated according to Mendelian inheritance patterns, which corroborates the insert stability demonstrated by NGS and independently establishes the nature of the T-DNA at a single chromosomal locus.

Bioinformatic analysis of the 5'and 3' flanking regions of the MON 94313 insert did not reveal any putative peptides that would cause safety concerns. No structurally and immunologically relevant similarities exist between the newly expressed proteins and known allergens, toxins, or other biologically active proteins that would raise a safety concern for human or animal health. No unintended changes, or loss of functions of specific genes were identified and it can be concluded that the molecular characterisation of MON 94313 does not indicate safety concerns.

Estimations of DMO, PAT, FT_T.1 and TDO expression levels in MON 94313 were provided for forage and grain tissues as well as for flower, used as surrogate for pollen.

Investigation of the potential risk associated with HGT show that the occurrence of HR in MON 94313 to humans, animals or micro-organisms is extremely unlikely.

Based on the above, it is concluded that the molecular characterisation of MON 94313 does not raise any safety concern and does not show any evidence of unintended changes in MON 94313.

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EXHIBIT K
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EXHIBIT L
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EXHIBIT M
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